Maladies chromosomiques constitutionnelles : corrélations génotype-phénotype

Carole Goumy

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par

GOUMY Carole

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Président :	Prof. Pierre SARDA
Directeur :	Prof. Philippe VAGO
Rapporteur :	Prof. Brigitte BENZACKEN
Rapporteur :	Dr Franck PELLESTOR
Examinateur :	Prof. Didier LEMERY
Examinateur :	Prof. Vincent SAPIN

Département de Cytologie Histologie Embryologie Cytogénétique
UMR CNRS, Clermont Université, INSERM U931 – GReD
Faculté de Médecine Clermont-Ferrand – Université d'Auvergne

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Abréviations

ADN : Acide DesoxyriboNucléique

AR : Acide Rétinoïque

ARN : Acide RiboNucléique

BAC : Bacterial Artificial Chromosome

CEP : Centromeric Enumeration Probe

CGH : Comparative Genomic Hybridization

CIA : Communication Inter-Auriculaire

CIV : Communication Inter-Ventriculaire

CN : Clarté Nucale

CNV : Copy Number Variation

DS : Déviation Standard

FISH : Fluorescence *in situ* par Hybridation

GTG : G-bands after Trypsin and Giemsa

HDC : Hernie Diaphragmatique Congénitale

IMG : Interruption médicale de Grossesse

Kb : Kilo base

LCR : Low Copy Repeats

LS : Larsen Syndrome

LSI : Locus Specific Identification

Mb : Mega base

MLPA : Multiplex Ligation Probe Amplification

NOR : Nucleolar Organiser Region

PCR : Polymerase Chain Reaction

QMPSF : Quantitative Multiplex PCR of Short Fluorescent fragments

RCIU : Retard de Croissance Intra-Utérin

RHG : Reverse Heat Giemsa

SA : Semaine d'Aménorrhée

YAC : Yeast Artificial Chromosome

Introduction générale

En 1958, le Professeur Jérôme Lejeune (1926-1994), à peine deux ans après que le nombre de chromosomes dans l'espèce humaine ait été établi à 46, fait, pour la première fois au monde, le lien entre un syndrome clinique avec retard mental et une anomalie chromosomique : la trisomie 21. Cette première corrélation entre une altération du phénotype et une anomalie chromosomique a donné naissance à une nouvelle discipline : la cytogénétique médicale. Cinq ans plus tard, la même équipe parisienne décrit un nouveau syndrome chromosomique : la maladie du cri du chat ou monosomie 5p et établi le concept de type et contretype selon lequel deux syndromes dus l'un à une monosomie et l'autre à une trisomie pour le même segment chromosomique s'opposent par leurs signes cliniques.

La cytogénétique médicale a pour objet la détection d'anomalies chromosomiques constitutionnelles ou acquises grâce à des techniques microscopiques ou moléculaires afin d'établir un diagnostic biologique et d'assurer un conseil génétique. Pour ce faire, elle étudie l'arrangement des gènes au niveau des chromosomes (recherche de remaniements chromosomiques) et quantifie le nombre de copies des gènes (délétions, duplications).

En cytogénétique médicale constitutionnelle, deux types de maladies chromosomiques sont décrits :

- les maladies dites déséquilibrées, caractérisées par une perte ou un gain en gènes ; elles sont responsables d'un handicap plus ou moins sévère associant de façon variable un retard mental et/ou des troubles comportementaux et/ou des malformations viscérales et/ou un syndrome dysmorphique.
- les maladies dites équilibrées, dans lesquelles tous les gènes sont présents, et en bonne quantité, mais disposés de façon anormale. Les translocations réciproques par exemple ne s'accompagnent d'aucune altération du phénotype chez les personnes porteuses mais peuvent être à l'origine de troubles de la reproduction et/ou se transmettre de façon déséquilibrée dans la descendance.

Nous montrerons dans ce travail à l'aide de plusieurs cas cliniques que la théorie initiale selon laquelle à une anomalie équilibrée correspond un phénotype « normal » et à une anomalie déséquilibré une altération du phénotype n'est pas toujours respectée.

Si les techniques chromosomiques de la cytogénétique permettent de détecter les anomalies du nombre des chromosomes et des remaniements de structure de grande taille, elles ne permettent pas de visualiser les remaniements d'une taille inférieure à 5-10 Mb,

niveau de résolution maximum du caryotype. Aussi, de nouvelles approches plus résolutives ont été développées. C'est ainsi que sont nées les techniques moléculaires de la cytogénétique : Fluorescence *In Situ* par Hybridation (FISH) dans les années 1980, Hybridation Génomique Comparative (CGH) sur métaphases dans les années 1990, CGH sur puces à ADN au début des années 2000 et récemment les techniques moléculaires de quantification génique : Quantitative Polymerase Chain Reaction (Q-PCR), Multiplex Labelling Probe Amplification (MLPA), Quantitative Multiplex PCR of Short Fragments (QMPSF)…

Aujourd'hui, la résolution en cytogénétique a atteint le niveau du gène, permettant d'affiner les corrélations génotype/phénotype et donc d'améliorer le conseil génétique des maladies chromosomiques (Vago, 2009).

Plus récemment, peu après l'achèvement du déchiffrage de la séquence du génome humain et notamment grâce à la technique d'Hybridation Génomique Comparative sur puce à ADN (CGH-array), des scientifiques ont publié des résultats venant bouleverser le consensus général selon lequel le génome varie relativement peu d'un individu à l'autre. En effet, de grands segments d'ADN de la taille d'un gène peuvent être présents en nombres différents de copies chez des individus « sains » (Redon *et al*, 2006 ; Komura *et al*, 2006 ; Carter, 2007 ; De Stahl *et al*, 2008 ; Jakobsson *et al*, 2008). La mise en évidence et la cartographie de ces variations/polymorphismes du nombre de copies de gènes (CNV ou CNP) a remis en cause le principe selon lequel à un génotype correspond un phénotype et inversement. Certains CNV sont associés à des gènes qui jouent un rôle dans la réponse immunitaire et dans le métabolisme lié à la détoxification ce qui pourrait expliquer notamment la variabilité inter-individus quant à la prédisposition à des maladies ou la réponse à des traitements (Weiss *et al*, 2008). D'autre CNV se sont avérés être à l'origine de maladies graves comme l'autisme ou d'une résistance à plusieurs virus dont le VIH (Sebat *et al*, 2007 ; Nakajima *et al*, 2008). A l'opposé, il existe des régions du génome dont le nombre de copies ne varie jamais, ce qui indique qu'un dosage fixe de cet ADN est indispensable à la vie (Redon *et al*, 2006). Enfin, chez certains individus sains, certains gènes sont absents, ce qui nous porte à nous interroger sur leur nécessité et leur fonction. La présence de ces CNV rend l'interprétation de nos résultats de cytogénétique délicate, particulièrement depuis l'utilisation de nouvelles techniques moléculaires de quantification génique.

Le premier objectif de ce mémoire est de montrer l'apport des diverses techniques moléculaires de cytogénétique pour le diagnostic clinique constitutionnel. Ces techniques de plus en plus résolutives nous permettent de mettre en évidence des anomalies

chromosomiques de petite taille, peu ou pas décrites dans la littérature et donc à phénotype souvent mal connu. L'interprétation de ces résultats en cas d'anomalie, notamment en prénatal, est donc délicate. En effet, même si des critères tels que la taille du déséquilibre, son caractère hérité ou non, la description de cas similaires dans la littérature nous permettent dans la majorité des cas de prévoir le phénotype et de faire un conseil génétique approprié, dans certains cas, le phénotype peut être atypique ou il peut exister une variabilité du phénotype pour une même anomalie chromosomique au sein d'une même famille ceci rendant le conseil génétique difficile.

Dans la deuxième partie de ce mémoire, nous discuterons à travers plusieurs cas cliniques publiés, les hypothèses pouvant expliquer de telles discordances génotype-phénotype. Ce travail montre la nécessité d'avoir recours aux techniques moléculaires de la cytogénétique pour mieux documenter les cas de discordance entre le caryotype et le phénotype observés.

Si un même génotype peut avoir une grande variabilité phénotypique, inversement, un même phénotype peut être associé à différents remaniements chromosomiques. Une des explications possible est que dans les différentes régions chromosomiques impliquées se trouvent des gènes appartenant à une même voie de signalisation. L'identification de ces gènes peut permettre de mieux comprendre la physiopathologie de certaines malformations et éventuellement de proposer des thérapeutiques ciblées. Pour explorer cette hypothèse, nous avons entrepris une étude à la fois génomique et transcriptomique en prenant comme modèle la hernie diaphragmatique congénitale et la voie de signalisation des rétinoïdes. Les résultats obtenus à ce jour sont présentés dans la troisième partie de ce mémoire.

1ère partie

1ère PARTIE : Techniques conventionnelles et moléculaires de cytogénétique et applications

I/ Techniques chromosomiques : le caryotype

Le caryotype est le classement par paire des chromosomes en métaphase ou prométaphase (caryotype haute résolution) d'un sujet en fonction de leur taille, de leur indice centromérique et de leur marquage en bande. Il peut être réalisé sur de nombreux types cellulaires et donc obtenu à partir de divers prélèvements biologiques comme le sang, le liquide amniotique ou les villosités choriales en prénatal mais également des prélèvements tissulaires comme la peau. Son obtention nécessite dans la majorité des cas une culture cellulaire dont la durée varie de 72h pour le sang à une à deux semaines pour les prélèvements réalisés en prénatal et les prélèvements cutanés. Ensuite la technique classique consiste à bloquer les cellules en métaphase grâce à la colchicine (poison du fuseau mitotique), réaliser un choc hypotonique pour faire gonfler et éclater les cellules et disperser le cytoplasme puis fixer et étaler la préparation cellulaire sur une lame afin d'obtenir une dispersion chromosomique satisfaisante. Enfin, des méthodes de dénaturation avant coloration par le GIEMSA permettent d'obtenir un marquage en bandes (GTG par dénaturation enzymatique, RHG par dénaturation thermique) c'est-à-dire une alternance, le long des chromosomes, de bandes transversales claires et sombres spécifique de chaque paire chromosomique. Il existe d'autres techniques de colorations spécifiques de segments chromosomiques comme les bandes C pour l'hétérochromatine constitutive (centromères et constrictions secondaires) ou l'imprégnation argentique pour les organisateurs nucléolaires (régions contenant les gènes des ARN ribosomiques ou NOR).

Les quatre critères à prendre en compte pour l'interprétation du caryotype sont :

- le nombre de chromosomes,
- la structure des chromosomes
- le caractère équilibré ou déséquilibré d'un éventuel remaniement,
- le caractère homogène ou en mosaïque des anomalies

Les indications du caryotype en constitutionnel sont nombreuses et peuvent être envisagées en fonction de l'âge du patient.

A la naissance : dysmorphie faciale / malformations / hypotonie / hypotrophie / ambiguïté sexuelle

Dans l'enfance : retard de développement psychomoteur / retard mental / troubles du comportement / dysmorphie / malformations

A la puberté : aménorrhée primaire / absence de développement pubertaire / anomalie du développement statural

Chez l'adulte : enquête familiale / pathologies de la reproduction et donneurs de gamètes / fausses couches ou morts fœtales *in utero* à répétition

En prénatal, il existe six indications prises en charge par l'assurance maladie :

- risque de trisomie 21 fœtale > 1/250, estimé par dépistage combiné du 1er trimestre ou dépistage séquentiel intégré du 2ème trimestre ou, à défaut, dépistage par marqueurs sériques du 2ème trimestre
- anomalies chromosomiques parentales
- antécédent de grossesse(s) avec caryotype anormal
- signes d'appel échographiques
- diagnostic de sexe fœtal pour les maladies liées à l'X
- âge maternel ≥ 38 ans (si la patiente n'a pu bénéficier d'aucun dépistage de la trisomie 21)

Dans la majorité des cas, l'anomalie chromosomique recherchée est une anomalie de nombre, visible au caryotype standard. Cependant, notamment en cas de syndrome polymalformatif, il peut s'agir de remaniements complexes parfois non visibles ou non interprétables de façon précise au caryotype standard et nécessitant le recours aux autres techniques moléculaires de la cytogénétique.

Le caryotype, s'il permet une vision globale du génome, possède une résolution maximale de 5 à 10 Mb. Cet examen est donc réalisé en première intention pour rechercher des anomalies de nombre ou de structure de grande taille mais en cas de phénotype fortement évocateur d'une anomalie chromosomique et d'absence d'anomalie au caryotype, il est nécessaire d'avoir recours à d'autres techniques plus résolutives.

II/ Techniques moléculaires fondées sur l'hybridation *in situ*

(Gouas et al, 2008, Publication n°1)

1) La Fluorescence *in situ* par Hybridation (FISH)

Les trois indications majeures de cette technique en constitutionnel pré et post-natal sont : 1) la recherche d'anomalies de nombre « en direct » (avant culture) sur noyaux interphasiques, 2) la caractérisation de remaniements de structure et 3) la recherche de microremaniements.

Les techniques d'hybridation *in situ* sont fondées sur la propriété de complémentarité des bases nucléotidiques. Une sonde dénaturée (ADN simple brin marqué) en solution va s'hybrider spécifiquement avec sa séquence cible (noyau interphasique / préparation chromosomique) après dénaturation (Fig. 1). Les sondes sont marquées par des fluorochromes et la lecture se fait au microscope à fluorescence grâce à l'utilisation de filtres spécifiques.

De nombreuses sondes sont commercialisées et prêtes à l'emploi pour la détection de syndromes cliniques connus. Les sondes existant dans le commerce ne couvrant qu'une petite partie du génome, il est parfois nécessaire de fabriquer des sondes « à façon » à partir de BAC (Bacterial Artificial Chromosome). Ces BAC sont choisis dans les banques de données du génome humain (http://genome.ucsc.edu/) et reçus sous la forme de cultures de bactéries recombinantes contenant la séquence ADN d'intérêt qui sera extraite et marquée (cf. protocole en annexe).

Cependant cette technique présente des limites, en particulier celle d'être une approche ciblée nécessitant une identification clinique ou cytogénétique préalable de l'anomalie à rechercher.

Différents types de sondes sont utilisés (Fig. 1) :

- Les sondes chromosomes spécifiques (centromériques ou locus spécifiques)

Elles sont utilisées pour rechercher des anomalies de nombre. Elles permettent de compter le nombre de chromosomes dans les noyaux interphasiques. L'avantage de ces sondes est qu'elles peuvent être utilisées « en direct », avant culture. La principale indication est le diagnostic prénatal en urgence des trisomies 13, 18, 21 et des aneuploïdies gonosomiques en cas de signes d'appel échographiques évocateurs (Eiben *et al*, 1999 ; Tepperberg *et al*, 2001).

Nous avons montré la faisabilité et l'intérêt d'une telle approche par FISH sur noyaux interphasiques à partir de prélèvements de villosités choriales ouvrant ainsi la voie d'un diagnostic prénatal chromosomique précoce (à partir de 11 SA) et rapide (moins de 24h) (Goumy et al, 2004, Publication n°2). Les deux indications à ce diagnostic prénatal précoce sont : 1) la présence de signes d'appel échographiques évocateurs d'une aneuploïdie, notamment une anomalie de l'épaisseur de la clarté nucale (CN) à l'échographie du premier trimestre ; 2) l'existence d'un remaniement de structure équilibré chez un des deux parents.

Une étude récente, portant sur 648 fœtus, montre une prévalence d'aneuploïdie de 15% pour une clarté nucale comprise entre le 95ème percentile et 3,4 mm, de 30% pour une CN entre 3,5 et 4,4 mm, de 47% entre 4,5 et 5,4 mm, de 63% entre 5,5 et 6,4 mm et de 65% lorsque la clarté nucale est supérieure ou égale à 6,5 mm (Bilardo *et al*, 2007). De plus, la sensibilité de la FISH en direct serait plus élevée lorsque le signe d'appel est de découverte précoce. En effet, une anomalie échographique majeure comme une pathologie de la clarté nucale décelée au premier trimestre de grossesse correspond le plus souvent à une anomalie chromosomique « majeure » comme une aneuploïdie, alors que des malformations isolées retrouvées à un stade plus tardif du développement peuvent correspondre à des anomalies chromosomiques plus fines et plus complexes ou a des pathologies non chromosomiques (Thein *et al*, 2000 ; Pergament *et al*, 2000).

En cas de remaniement parental, grâce à un choix judicieux de 2 à 3 sondes ciblant les chromosomes impliqués dans le remaniement, il est possible, de statuer sur le caractère équilibré ou déséquilibré du fœtus dès le lendemain du prélèvement.

Le principal avantage de cette approche est de pouvoir discuter, en cas de déséquilibre, d'une interruption médicale de grossesse avant 14 semaines d'aménorrhée, par simple aspiration, et donc moins traumatisante pour la patiente.

- Les sondes locus spécifiques (LSI) **et subtélomériques**

Elles sont utilisées en cytogénétique constitutionnelle pré- et postnatale pour rechercher des syndromes microdélétionnels, subtélomériques comme le syndrome de Wolf Hirshhorn (MIM 194190) ou interstitiels, comme le syndrome de Di George (MIM 188400). Une vingtaine de microremaniements dont la fréquence cumulée est de 1/1000 naissances peuvent être détectée par FISH. Ces remaniements récurrents sont dus à la présence de séquences d'ADN répétées en tandem appelés duplicons ou LCR (Low Copy Repeats) ayant une grande homologie et donc susceptibles d'entrainer des recombinaisons homologues non alléliques pendant la méiose. Ces LCR représenteraient 5% du génome ce qui renforce l'hypothèse selon laquelle la fréquence de ces microremaniements et des syndromes associés est sous estimée. On sait aujourd'hui que les microremaniements subtélomériques déséquilibrés sont à l'origine de près de 7% des retards mentaux idiopathiques (Knight *et al*, 1999 ; Koolen *et al*, 2004 ; Menten *et al*, 2006).

Les approches ciblées par FISH ne sont possibles que lorsque le phénotype est caractéristique et l'anomalie moléculaire récurrente : microdélétions interstitielles « classiques » (syndrome de Di George, syndrome de Williams…) ou remaniements subtélomériques (1p36, 2q37, Wolf Hirshhorn, 22q13.3) (De Vries *et al*, 2001).

Les sondes subtélomériques sont également utilisées pour rechercher des translocations équilibrées -dans le cadre de troubles de la reproduction essentiellement-, ou déséquilibrées -dans le cadre de l'exploration de troubles du développement notamment-, de petite taille, invisibles au caryotype standard (Granzow *et al*, 2000 ; Popp *et al*, 2002 ; Novelli *et al*, 2004). Ces sondes sont alors regroupées en « kits » permettant en une seule analyse, l'exploration des 46 extrémités subtélomériques.

- Les sondes pantélomériques

Elles ciblent les séquences répétées télomériques communes à toutes les extrémités des 23 paires chromosomiques. Elles sont utilisées en constitutionnel pour la mise en évidence des chromosomes en anneaux.

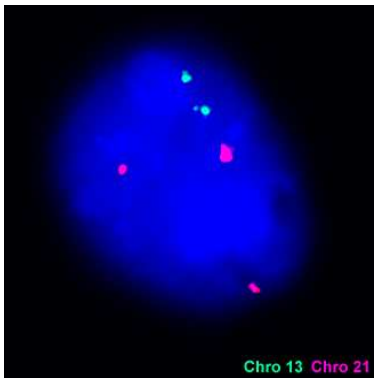
- **Les sondes de peinture chromosomique** (WCP pour Whole Chromosome Paint)

L'utilisation combinée d'un ensemble de sondes spécifiques d'une paire chromosomique donnée permet d'obtenir un marquage de tout le matériel chromosomique appartenant à cette paire, même située sur un autre chromosome. Les sondes correspondant aux 23 paires chromosomiques sont commercialisées. Ces sondes permettent essentiellement de caractériser une anomalie chromosomique mise en évidence par une autre technique

- La M-FISH

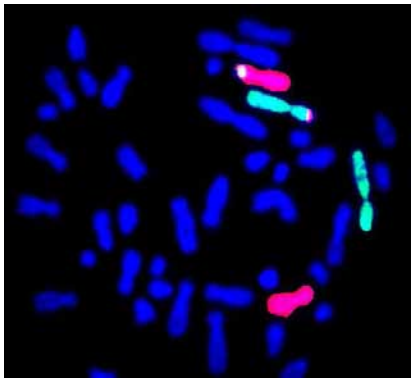
Le caryotype multicouleur (M-FISH), contrairement à la FISH, permet une analyse globale du génome. Cette technique nécessite l'obtention de métaphases. Elle consiste à peindre spécifiquement chaque paire chromosomique d'une couleur différente à l'aide de sondes réparties tout au long du génome. Pour une paire donnée, les différentes sondes spécifiques de cette paire sont couplées à une combinaison unique de 1 à 3 fluorochromes parmi les 5 utilisés, ceci générant une couleur propre à chaque paire chromosomique (Fig. 1). Cette technique permet l'identification de chromosomes dérivés ou marqueurs et de remaniements complexes (Schröck *et al*, 1997 ; Ning *et al*, 1999).

FIGURE 1 : La FISH : les différents types de sondes



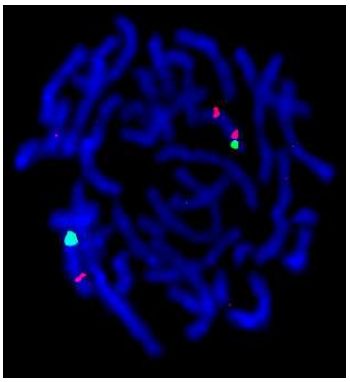
FISH
chromosome spécifique

Compter nombre chromosomes



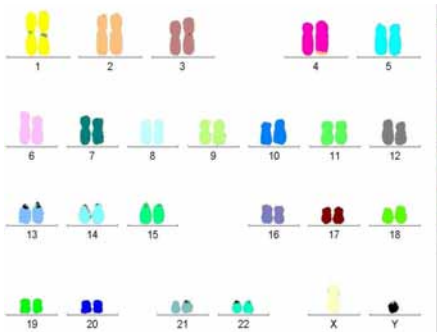
FISH
peinture chromosomique

Caractériser remaniements



FISH
locus spécifique

Micro-remaniements



M-FISH

Identifier marqueurs et chromosomes dérivés

M-FISH (caryotype multicouleur)

CHR.	FITC	SpeOrange	Texas Red	Cy5	DEAC
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
X					
Y					

2) L'Hybridation Génomique Comparative (CGH) sur métaphases et sur puces à ADN (CGH array)

Le principe de ces deux techniques est identique. Une hybridation compétitive est réalisée entre deux ADN, celui du patient et celui d'un témoin. Ces ADN, marqués par 2 fluorochromes différents (cyanine 5 = rouge et cyanine 3 = vert), vont être hybridés en compétition soit sur des métaphases d'un sujet « normal » pour la CGH sur métaphases, soit sur des fragments d'ADN génomique (BAC ou oligonucléotides) appelés clones pour la CGH-array (Fig. 2). Une différence dans le nombre de copies d'une séquence d'ADN entre le génome du patient et celui de référence modifiera le rapport « fluorescence vert / fluorescence rouge » (Fig. 2).

Pour la CGH sur métaphases, après la saisie et le classement d'une vingtaine de métaphases, l'acquisition des images est faite pour chaque fluorochrome. Un logiciel d'analyse d'images calcule le rapport des intensités de fluorescence pixel par pixel tout au long des chromosomes. Un profil est ainsi obtenu pour chaque chromosome et mis en regard d'un idéogramme permettant la localisation cytogénétique d'un éventuel déséquilibre. Le principal avantage de cette technique est qu'elle permet une exploration pangénomique même si ce n'est qu'à une faible résolution (≈ 5 Mb).

Pour la CGH array, les lames sont lues par un scanner laser. Des logiciels permettent l'acquisition des images et l'analyse des données. Le rapport de fluorescence est calculé au niveau de chaque clone et les résultats sont rendus sous forme de graphes, ce qui facilite l'interprétation (Fiegler *et al*, 2003 ; Mantripragada *et al*, 2004).

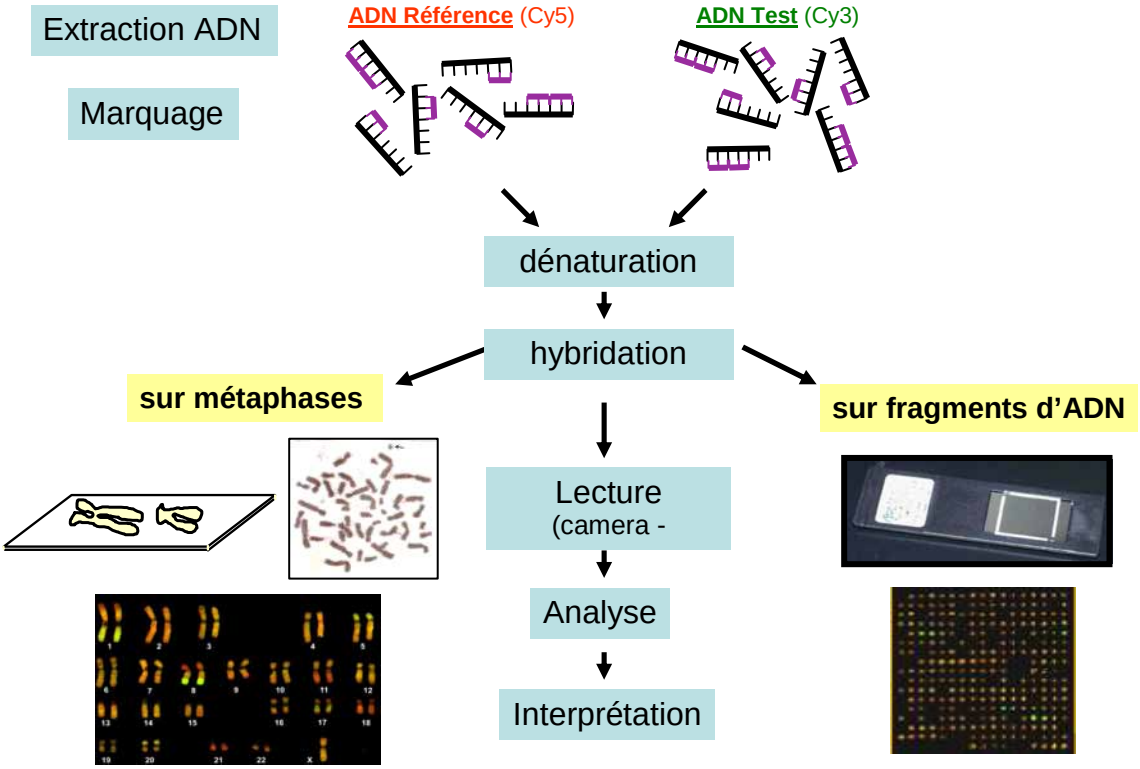
Ces deux techniques permettent donc de détecter et, simultanément, de localiser sur le génome des pertes ou des gains en ADN génomique. L'avantage de ces techniques est qu'elles ne nécessitent pas l'obtention de métaphase et donc de culture cellulaire.

La résolution de la CGH sur métaphases est d'environ 5 Mb. Elle permet d'identifier des déséquilibres non visibles au caryotype standard mais sa résolution ne permet pas de détecter les remaniements de petite taille observés notamment dans les syndromes microdélétionnels subtélomériques ou interstitiels.

La couverture génomique de la CGH array dépend de sa densité en clone ; pour les puces « pangénomiques » la résolution va dépendre de la distance séparant deux clones : elle peut atteindre quelques Kb. Elle est tout à fait indiquée pour la recherche des microremaniements en pathologie constitutionnelle. Certaines puces dites ciblées permettent par exemple l'exploration des régions subtélomériques ou impliquées dans les principaux remaniements connus et sont spécialement dédiées au diagnostic clinique chez

des patients avec retard des acquisitions / retard mental / dysmorphie faciale (Veltman *et al*, 2002 ; Van Karnebeek *et al*, 2002 ; Harada *et al*, 2004 ; Shaw-Smith *et al*, 2004 ; Schoumans *et al*, 2005 ; Shaffer *et al*, 2007).

FIGURE 2 : Principe de la CGH sur métaphase et sur puce à ADN



La CGH array, en mettant en évidence des microremaniements récurrents et en réduisant la taille des régions d'intérêt, a permis de faire des corrélations génotype-phénotype très précises et d'identifier des gènes candidats dans certaines pathologies. Par exemple, la mise en évidence d'une microdélétion en 8q12 chez deux patients atteints du syndrome CHARGE (colobome, anomalie cardiaque, atrésie des choanes, retard de croissance, anomalies génitales et anomalies des oreilles) a permis d'identifier le gène CHD7 comme responsable de cette affection (Vissers *et al*, 2004). Par une même approche, à l'aide d'une puce dédiée au chromosome X, le gène ZNF674 a été découvert grâce à la mise en évidence d'une délétion de 1Mb en Xp11 et décrit comme un des gènes responsable de retard mental lié à l'X (Lugtenberg *et al*, 2006). Enfin il existe des puces très résolutives ne concernant qu'une petite région du génome et permettant de faire des corrélations génotype/phénotype très poussées comme celle décrite par Yu *et al* possédant 97 clones dans la région 1p36 afin de caractériser de façon très précise la localisation des

points de cassure chez 25 patients porteurs d'une microdélétion 1p36 (Yu *et al*, 2003). Le principal avantage de cette technique est donc sa grande modularité puisque l'on peut concevoir des puces « à façon » répondant à une problématique précise.

La difficulté essentielle, surtout avec les puces pangénomiques très résolutives, est de pouvoir déterminer le caractère pathologique ou non (polymorphisme dans la population) des déséquilibres ainsi mis en évidence (Rosenberg *et al*, 2006). En effet, en 2004, Iafrate et al, ont explorés avec une puce commerciale 39 sujets considérés comme phénotypiquement normaux et mis en évidence plus de 200 clones polymorphes avec une moyenne de 12,5 par personne (Iafrate *et al*, 2004). Cette étude souligne les difficultés d'interprétation liées à la présence de polymorphismes, d'autant plus importantes que la puce est résolutive. Pour ces raisons, les laboratoires utilisent actuellement la CGH array comme technique de dépistage et non de diagnostic. Ainsi toute anomalie mise en évidence par cette technique devra être confirmée par d'autres techniques moléculaires de cytogénétique (FISH ou PCR quantitative) et, surtout, son implication dans l'altération du phénotype devra être établie (consultations des bases de données, en particulier celles recensant les CNV ; recherche de la présence éventuelle de l'anomalie chez les parents).

III/ Techniques moléculaires fondées sur la PCR (Gouas et al, 2008, Publication n°1)

1) La MLPA (Multiplex Ligation Probe Amplification)

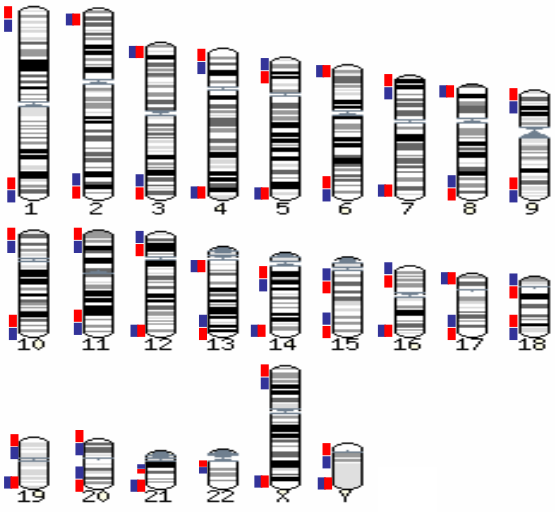
Cette technique, utilisée en routine dans notre service, est dotée d'une grande sensibilité et possède une grande capacité de multiplexage : jusqu'à 46 locus peuvent être analysés dans une même réaction. Elle a l'avantage de ne nécessiter qu'une faible quantité d'ADN (20 ng), ce dernier pouvant être extrait à partir de divers échantillons biologiques (sang, liquide amniotique, villosités choriales, tissus…). Cette technique est, de plus, une alternative sensible, rapide et peu onéreuse comparée aux techniques fondées sur l'hybridation *in situ*.

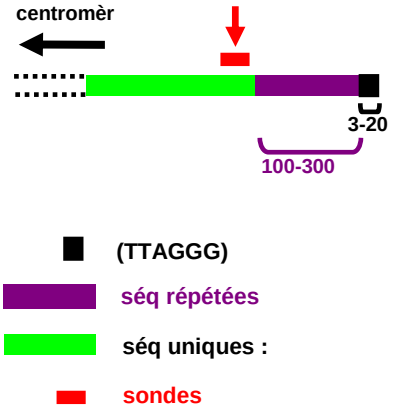
Le principe de la MLPA repose sur l'amplification simultanée par PCR de différentes sondes hybridées aux séquences du génome d'intérêt grâce à une seule paire d'amorces oligonucléotidiques fluorescentes (Fig. 3). L'intensité de fluorescence d'un produit de PCR (amplicon = amplification d'une région d'intérêt) reflète le nombre de copies de séquence présentes initialement.

FIGURE 3 : La MLPA

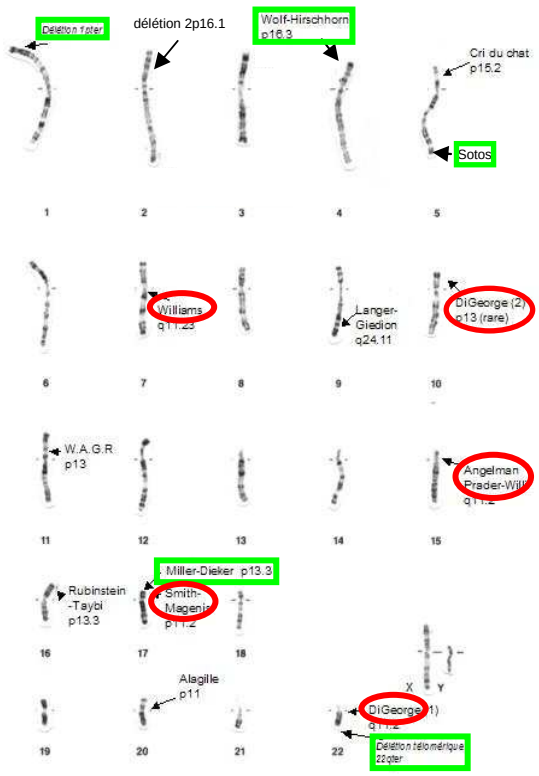
MLPA régions subtélomériques

46 extrémités explorées
par 2 kits





Microremaniements syndromiques

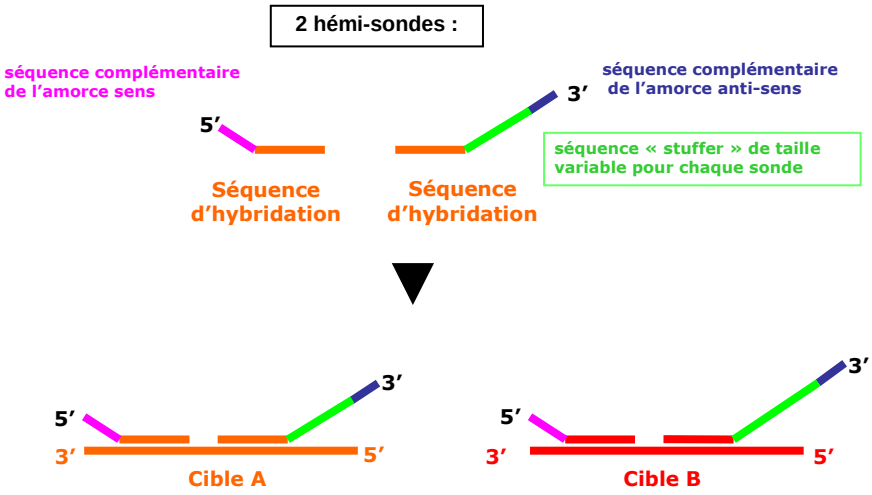


Syndrome microdélétionnel 1p36	TNFRSF4
	GABRD
	GNB1
Syndrome microdélétionnel 2p16.1	FANCL (PHF9)
	REL
Syndrome microdélétionnel 3q29	DLG1
	DLG1
Syndrome de Wolf-Hirschhorn, 4p16.3	LETM1
	WHSC1
Région du Cri du Chat, 5p15	CRR9 (=CLPTM1L)
	TERT
Syndrome de Sotos, 5q35.3	NSD1, exon17
	NSD1, exon22
Syndrome de Williams-Beuren, 7q11.23	ELN, exon 1
	ELN, exon 20
	LIMK1
Syndrome de Langer-Giedion, 8q24.12	TRPS1
	EIF3S3
Syndrome microdélétionnel 9q22.3	TGFBR1, exon 7
	TGFBR1, exon 8
DiGeorge, région 2, 10p14	GATA3
	Hs.538604
Syndrome WAGR, 11p13	PAX6, exon 5
Syndrome de Prader-Willi/Angelman, 15q12	UBE3A
	NDN
	SNRPN
	SNRPN
Syndrome délétionel 15q24	SEMA7A, exon 8
	CYP1A1, exon 2
Syndrome de Rubinstein-Taybi, 16p13.3	CREBBP
Région Miller-Dieker, 17p13.3	PAFAH1B1, exon 7
	PAFAH1B1, exon 3
Syndrome de Smith-Magenis, 17p11.2	LRRC48
	LLGL1
	RAI1
Syndrome microdélétionel NF1, 17q11.2	NF1, exon 12
	NF1, exon 20
Syndrome microdélétionel 17q21.31	MAPT, exon 11
	MAPT, exon 13
	CRHR1, exon8
Syndrome de DiGeorge, 22q11.21	CLDN5, région-AB
	GP1BB, région-AB
	SNAP29, région-CD
Syndrome de Phelan-McDermid, 22q13	SHANK3
	SHANK3
Syndrome de RETT, Syndrome de duplication de MECP2, Xq28	MECP2, exon 4
	MECP2, exon 4
	MECP2, exon 1

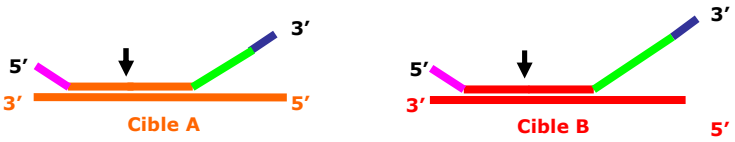
21 syndromes explorés
1 à 3 sondes par syndrome

Principe de la MLPA

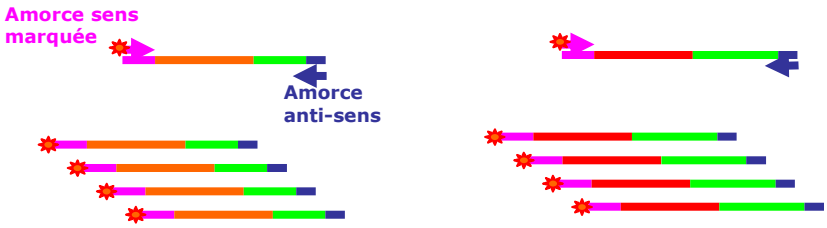
1. Hybridation des sondes



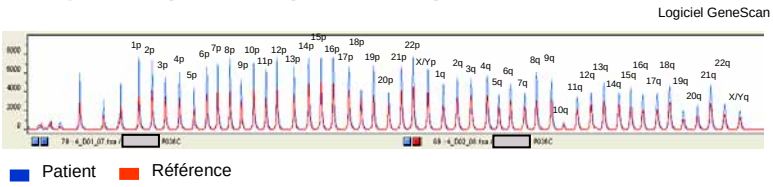
2. Ligation des sondes hybridées par la Ligase 65



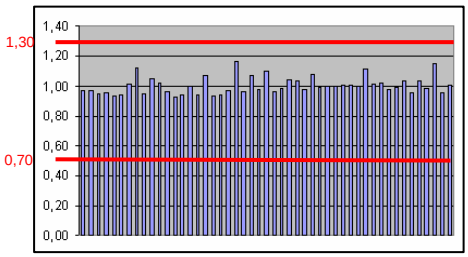
3. Amplification des sondes liées par PCR avec un couple unique d'amorces



4. Séparation par électrophorèse à capillaires



5. Histogramme des rapports d'intensité de fluorescence après normalisation et quantification



Chaque pic obtenu après séparation électrophorétique correspond à l'intensité de fluorescence pour un produit de PCR donné et reflète la quantité initiale de la séquence génomique cible. La quantification génomique repose sur la comparaison de l'intensité de fluorescence de chacun des amplicons du patient à celles obtenues chez un individu contrôle.

Trois kits commerciaux (MRC-Holland, Pays-Bas) sont utilisés dans le service (Protocole en annexe). Les kits P036D et P070 pour l'exploration des régions subtélomériques, et le kit P245 pour l'exploration de 21 régions chromosomiques impliquées dans les principaux microremaniements responsable de retards mentaux (MRS MLPA).

Cette technique est utilisée comme technique de criblage et tout résultat positif de MLPA doit être vérifié par FISH ou par une autre approche de quantification génique.

Il est possible d'appliquer la MLPA de façon très ciblée avec une haute résolution pour une caractérisation très fine d'un microremaniement. Jalali GR *et al* (2008) ont par exemple analysé 363 microdélétions 22q11 et mis en évidence de nouveaux variants avec notamment des points de cassures différents de ceux décrits dans les délétions les plus communes et dans quelques la présence de duplications associées. En cytogénétique constitutionnelle, la MLPA est surtout utilisée dans l'exploration des retards mentaux idiopathiques (Koolen *et al*, 2004 ; Rooms *et al*, 2006 ; Kirchhoff *et al*, 2007). En prénatal, la seule étude publiée concernait une analyse des régions subtélomérique chez des fœtus avec signes d'appels échographiques, mais la MLPA avait été réalisée en postnatal et n'avait donc pas influencé le conseil génétique durant la grossesse (Faas *et al*, 2008).

Nos résultats de MLPA en prénatal ont fait l'objet d'une publication soumise dans Journal of Medical Genetics (publication n°3). Nous avons mis en évidence 4 déséquilibres cryptiques sur les 63 fœtus explorés : une délétion 18pter/duplication 5pter, une délétion 9pter et une délétion 15q11q13 grâce aux kits subtélomériques et une délétion 22q11 à présentation échographique atypique grâce au kit MRS MLPA, soit un taux de détection de 6.3%. Deux grossesses ont été interrompues suite aux résultats de MLPA. Ces résultats montrent l'intérêt et la faisabilité de cette technique en prénatal.

2) La QMPSF (Quantitative Multiplex PCR of Short Fluorescent Fragments)

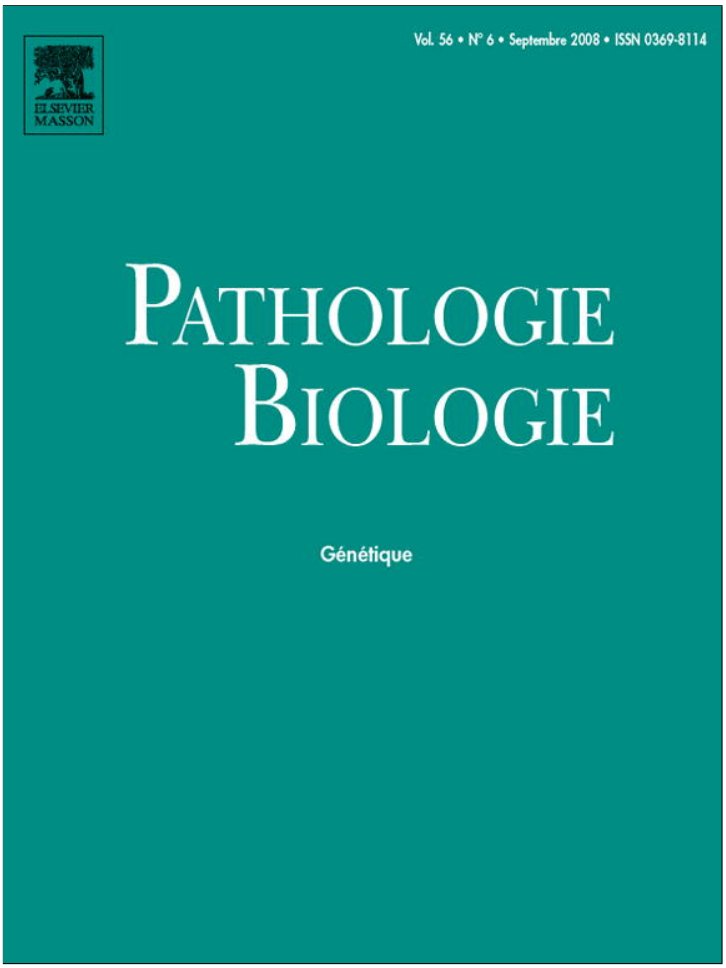
La QMPSF est une méthode semi-quantitative qui permet l'amplification simultanée par PCR multiplex de fragments génomiques cibles, généralement des exons de gènes, de 160 à 300 pb (séquences d'intérêt).

Le principe de cette technique repose sur 4 points :

- des amplicons de petite taille (<300 pb) sont générés pour chaque exon grâce à deux amorces dont l'une est marquée par un fluorochrome,
- la PCR est réalisée avec un nombre limité de cycles (en général inférieur ou égal à 21) pour se situer dans la phase exponentielle de l'amplification,
- après migration des produits de PCR sur séquenceur, l'analyse des chromatogrammes est basée sur la comparaison de l'intensité de fluorescence d'un même pic entre deux échantillons distincts après superposition informatique des chromatogrammes.

Cette technique permet l'analyse d'une douzaine de gènes de façon simultanée sur de grandes séries de patients (Saugier-Veber *et al*, 2006).

Publications n° 1 à 3



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Chorionic villus sampling (CVS) and fluorescence *in situ* hybridization (FISH) for a rapid first-trimester prenatal diagnosis

Carole Goumy[1]*, Marie-Noëlle Bonnet-Dupeyron[1], Yoan Cherasse[1], Hélène Laurichesse[2], Jean-Yves Jaffray[1], Gisèle Lacroute[1], Aimé Geneix[1], Didier Lemery[2] and Philippe Vago[1]

[1]*Cytogénétique Médicale, Faculté de Médecine/CHU, Clermont-Ferrand, France*
[2]*Médecine Fœtale, CHU, Clermont-Ferrand, France*

Objectives Early diagnosis of unbalanced chromosomal abnormalities can be crucial in minimizing the trauma caused by an elective abortion. Chorionic villus sampling (CVS) can be performed from 9 weeks of gestation. However, two major problems are encountered in fetal karyotyping using cultured cells from chorionic villi: the relatively slow growth of these cells in culture, which delays the diagnosis, and the occurrence of maternal cell contamination (MCC). With FISH, a result can be obtained within 24 h, and, as no cell culturing is involved, the problem of MCC is minimized.

Methods Thirty-two women undergoing CVS between 9 and 12 weeks of gestation were offered FISH analysis in addition to the standard chromosome analysis.

Results FISH was informative in all of the cases tested. Eleven aneuploidies were detected in cases of hygroma or abnormal nuchal translucency and two out of four fetuses from parental translocation were unbalanced. The decision to perform early termination of these chromosomally abnormal pregnancies was based on FISH results and ultrasound abnormalities, without waiting for karyotype results.

Conclusion The present study confirms that the association of FISH and CVS allows a rapid and early prenatal diagnosis, and emphasizes that this association is of great benefit in cases of known parental balanced translocation or when hygroma is detected by ultrasonography. Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS: chorionic villus sampling; FISH; first-trimester prenatal diagnosis

INTRODUCTION

Interphase fluorescence *in situ* hybridization (FISH) is now the standard procedure for the rapid detection of chromosomal aneuploidy from direct preparations of amniocytes or chorionic villi cells (Bryndorf *et al*., 1996; Eiben *et al*., 1998; Jalal *et al*., 1998; Bryndorf *et al*., 2000; Lewin *et al*., 2000; Pergament *et al*., 2000; Quilter *et al*., 2001; Tepperberg *et al*., 2001; Weremowicz *et al*., 2001; Witters *et al*., 2002). Interphase FISH with a specific probe set for familial rearrangements also allows rapid exclusion of unbalanced translocation in the fetus of a balanced translocation carrier (Cotter and Musci, 2001; Kilby *et al*., 2001; Pettenati *et al*., 2002). Amniocentesis is limited by the gestational age at which it can be performed (not before 13 weeks of gestation). On the other hand, chorionic villus sampling (CVS) can be performed as soon as the first trimester of pregnancy, and therefore is an effective method for early prenatal diagnosis, particularly appropriate for high-risk pregnancies (Kennerknecht *et al*., 1993; Brambati *et al*., 1998; Brun *et al*., 2003). However, obtaining a result from cultured chorionic villi cells may take up to two weeks, whereas a FISH assay can easily be completed in less than 24 h. If prenatal diagnosis results were obtained in the first trimester of chromosomally abnormal pregnancies, midtrimester abortion could be replaced by early termination using simple suction curettage, which involves much less risk for the mother, and is also more willingly accepted.

In the present study, we evaluated the clinical relevance of rapid FISH chromosome investigation in 32 uncultured chorionic villus samples taken in first-trimester pregnancies. The aim was to determine when to offer the association CVS + FISH and what its clinical applications are.

MATERIAL AND METHODS

Patients

Over a one-year period, 32 women undergoing chorionic villus sampling between 9 and 12 weeks of gestation were offered FISH analysis in addition to the obligatory conventional chromosome analysis. The indications were abnormal ultrasound findings (25 cases), maternal age >40 years (3 cases) and parental balanced translocation (4 cases). In these last cases, the parental karyotype was reviewed prior to selecting the probe set. Before the

*Correspondence to: Dr Carole Goumy, Service de Cytogénétique Médicale, Faculté de Médecine, Clermont-Ferrand Cedex, France.
E-mail: carole.goumy@u-clermont1.fr

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Revised: 5 January 2004
Accepted: 1 December 2003

study, interphasic FISH was performed on eight amniotic control samples to test the efficacy of the procedure in cases of parental translocation (Table 1).

Slide preparation

At their arrival in the laboratory, the chorionic villus samples were placed in a petri dish and covered with RPMI medium. Any maternal or anomalous material was removed by careful dissection under an inverted microscope. Sorted chorionic villi were exposed for 20 min to warm hypotonic solution (trisodium citrate 1% at 37 °C) and then fixed three times at room temperature with ethanol/glacial acetic acid (3 : 1) for 15 min each. After fixation, villi fragments were placed in the ejection medium (1 part distilled water: 1 part acetic acid) for 5 to 10 min and dissected with fine needles under a microscope. A drop of the cell suspension was placed on each end of a cold slide. The slides were dried at 37 °C for 2 h, placed in RNAse solution (0.1 mg/mL) at 37 °C for 30 min and washed in distilled water. They were then put in a pepsine solution (0.1 mg/mL) for 5 min. After washing in PBS for 5 min, the slides were allowed to air dry, were dehydrated in an ethanol series (70%, 90%, 100%) for two minutes each, and air dried again.

Probe hybridization and detection

FISH probes, all purchased from Vysis (ABBOTT, Rungis, France), were those of the AneuVysion™ Assay Kit and a specific set in cases of parental translocation (Locus Specific Identification probes (LSI), Chromosome Enumeration Probes (CEP) or Subtelomere probes (TEL), see Table 3 for details). Hybridization was performed according to the manufacturer's recommendations.

The same method was used for the translocation-specific probes, except that the denaturing temperature was lower (68 °C for 5 min).

Microscopic evaluation was performed using a Leica epifluorescence microscope with a filter set for Spectrum Orange, Spectrum Green and Spectrum Aqua. A minimum of 50 interphasic nuclei were analysed per patient for each probe. The criteria used for interpretation were as follows:

1. In cases in which <10% aneuploid nuclei were observed, euploidy was assumed.
2. Whenever >60% aneuploid nuclei were obtained, the case was classified as aneuploid.
3. If 10 to 60% aneuploid nuclei were found, the case was more intensively studied: up to 100 nuclei were scored, and routine cytogenetic analysis was performed on a larger number of metaphases.
4. If the distance between two spots was less than the width of two of these spots, the signal was considered as a split signal and was scored as one.

Conventional chromosome analysis

The standard karyotypes were performed by standard methods (Smidt-Jensen *et al*., 1989).

RESULTS

Table 1 gives the results of the FISH investigations on amniotic fluid for eight parental balanced translocations. All the FISH results were confirmed by the karyotype.

Table 2 summarizes the indications for testing and the informative results of AneuVysion™ in the 28 women for whom the indication was not a parental translocation.

The rate of chromosomal disorders detected was related to the indication for which the women were referred. First-trimester abnormal ultrasound was the most common indication for performing CVS and FISH. Informative FISH results were obtained before 12 weeks of gestation for all the pregnancies tested, and the sensitivity of the assay to detect trisomy 13, 18 and 21 and monosomy X was 100%. In one case, the amount of material was insufficient to carry out metaphase chromosome analysis but was sufficient for FISH analysis.

The incidence of abnormalities in this study (40.5%, 13/32) was very high and consistent with the fact that most tests were performed following abnormal ultrasound findings suggestive of those specific chromosomal disorders. In cases of abnormal nuchal translucency or hygroma, we observed 50% abnormalities. In contrast, when the indications were ultrasound abnormalities other than hygroma or advanced maternal age, no abnormal result was found.

FISH analysis was also performed to determine the balanced or unbalanced status of a fetus when one of

Table 1—FISH and karyotype results of amniotic control sample in eight cases of parental translocation

N°	Parental translocation	DNA probes[a]	FISH results	Karyotype
1	46,XX,t(10;12)(q11;q23)	CEP 10, CEP 12	Normal	46,XX,t(10;12)(q11;q23)mat
2	46,XX,t(14;22)(q12;q11.2)	LSI 14q32, LSI 22q11, LSI 22q13	Normal	46,XY
3	46,XX,t(3;12)(p21;p13)	CEP 12, CEP 3, TEL 3p	TEL 3p:3 spots	46,XX,der(12)t(3;12)(p21;p13)mat
4	46,XX,t(11;22)(qter;q12)	CEP 11, LSI 22q11, LSI 22q13	XY, Normal	46,XY,t(11;22)(qter;q12)mat
5	45,XX,der(14;21)(q10;q10)	AneuVysion	XY, Normal	45,XY,der(14;21)(q10;q10)mat
6	45,XX,der(13;14)(q10;q10)	AneuVysion	XY, Normal	45,XY,der(13;14)(q10;q10)mat
7	45,XX,der(13;14)(q10;q10)	AneuVysion	XX, Normal	46,XX
8	46,XY,t(1;12)(q31;p12)	TEL 1q, CEP 12, TEL 12p	XX, Normal	46,XX,t(1;12)(q31;p12)pat

[a] All probes provided by Vysis Company.

the parents carried a balanced translocation (Table 3). In all four cases, results were confirmed by standard cytogenetic analysis. In the woman with the 10;21 translocation, there were two copies of each probe of the chromosome involved (Figure 1a,b,c). These analyses suggested that the fetus had either a normal chromosome complement or a balanced reciprocal translocation. FISH using LSI 21 and TEL 10p probes on one direct metaphase showed that the fetus bore the maternal 10;21 translocation (Figure 1d and e). Standard karyotype confirmed that the fetus carried the balanced reciprocal translocation (Figure 1).

In contrast, in the woman with the 7;21 translocation, an abnormal result was obtained with three copies of the Vysis LSI 21 probe on direct preparations (Figure 2a) for an XY fetus, which is suggestive of an extra copy of the 21q region. We then performed standard karyotype after long-term culture, which showed maternal contamination with 100% 46,XX,t(7;21)(q35;q22) metaphases (the fetus was XY). FISH on slides from long-term cultured chorionic tissue showed about 10 Y nuclei (Figure 2b) and a Y metaphase (Figure 2c), all with three copies of the LSI 21 probe, among the maternal cells showing two copies of LSI 21 probe. Again, on slides from long-term culture, with WCP 7 and WCP 21 probes, on two XY metaphases, FISH suggested that the fetus had an unbalanced derived chromosome 7 [der(7)t(7;21)(q35;q22)] (Figure 2d).

In the case of the 5;10 paternal translocation, FISH on direct preparations with the 5p15, CEN 10 and TEL 10p probes showed that the fetus had a partial 5p monosomy and a partial 10p trisomy (Figure 3a,b,c). The karyotype was 46,XX,der(5)t(5;10)(p12;p12)pat, which was consistent with the FISH results.

Table 2—AneuVysion™ and karyotype results of 28 chorionic villus samples performed for sonographic anomalies or maternal age >40 years

N°	Indication	GA[a]	AneuVysion[b]	Mesenchyme cells karyotype
1	Increased nuchal translucency	10	XY, Normal	46,XY
2	or	11	XY, Normal	46,XY
3	hygroma colli	10	**X0, Turner**	45,X
4		12	**XY, T18**	46,XX (maternal cells)
5		10	XY, Normal	46,XY
6		11	**XX, T21**	46,XX,der(21;21)(q10;q10),+21
7		9	**XY, T21**	no karyotype
8		11	XY, Normal	46,XY
9		11	**X0, Turner**	**45,X0**
10		12	**XX, T13**	**47,XX,+13**
11		11	**XY, T21**	**47,XY,+21**
12		10	XY, Normal	46,XY
13		9	XX, Normal	46,XX
14		12	**XY, T21**	**47,XY,+21**
15		10	**XY, T21**	**47,XY,+21**
16		11	**XX, T18**	**47,XX,+18**
17		11	XX, Normal	46,XX
18		11	XX, Normal	46,XX
19		10	XX, Normal	46,XX
20		11	XX, Normal	46,XX
21		11	XY, Normal	46,XY
22		11	**XY, T21**	**47,XY,+21**
23	Brain malformation	10	XX, Normal	46,XX
24	Digestive malformation	11	XY, Normal	46,XY
25	Kidney malformation	10	XY, Normal	46,XY
26	Maternal age >40 years	11	XY, Normal	46,XY
27		11	XY, Normal	46,XY
28		10	XX, Normal	46,XX

[a] GA, gestational age (weeks).
[b] Performed on trophoblast cells.

Table 3—FISH with specific probes and karyotype results of the 4 chorionic villus samples in cases of parental translocation

N°	Indication	GA	AneuVysion	Specific FISH[a]	FISH conclusion	Karyotype
29	t(7;21)mat	9	**XY; T21**		Unbalanced	46,XX,t(7;21)(q35;q22) maternal cells
30	t(5;10) pat	10	XX; Normal	LSI 5p15, CEP 10, TEL 10p	Unbalanced	46,XX,der(5)t(5;10)(p12;p12)pat
31	t(10;21) mat	9	XY; Normal	LSI 21, CEP 10, TEL 10p	Balanced/translocated	46,XY,t(10;21)(p12;q11)mat
32	t(4;17)mat	11	XX; Normal	TEL 4p, TEL 4q, TEL 17q	Balanced/translocated	46,XX,t(4;17)(p16.2;q25)mat

[a] All probes provided by Vysis Company.

Prenatal detection of cryptic rearrangements by MLPA in fetuses with ultrasound abnormalities

Short running title: MLPA in prenatal diagnosis.

Goumy, Carole MD and Gouas, Laetitia PhD[a]; Pebrel-Richard, Céline MD; Véronèse, Lauren MD; Eymard-Pierre, Eleonore; Debost-Legrand, Anne; Haoud, Khadidja; Tchirkov, Andrei MD, PhD; Vago, Philippe MD, PhD.

Univ Clermont 1, UFR Médecine, Histologie Embryologie Cytogénétique, Clermont-Ferrand, F-63001 France; CHU Clermont-Ferrand, Cytogénétique Médicale, Clermont-Ferrand, F-63003 France.

[a] These authors contributed equally to the work

Correspondence to:

Doctor Carole GOUMY

Cytogénétique Médicale

Faculté de Médecine – BP 38

63001 Clermont-Ferrand Cedex – France

Tel: +33 (0)4 73 17 81 00

Fax: +33 (0)4 73 26 91 82

E-mail: cgoumy@chu-clermontferrand.fr

Conflict of interest

The authors declare no conflict of interest.

Abstract

Purpose: Congenital malformations are a major cause of morbidity and mortality in newborn infants, and genomic imbalances are a significant component of their etiology. The aim of this study was to evaluate the ability of prenatal MLPA (Multiplex Ligation Probe Amplification) screening to detect cryptic chromosomal imbalances in fetuses with ultrasound abnormalities of unknown etiology.

Methods: MLPA was performed with 3 separate sets of probes: two for subtelomeric regions and one for mental retardation syndrome loci. Sixty-one fetuses with significant ultrasound anomalies and normal karyotype at a minimum of 400-band resolution were tested between January 2007 and January 2009.

Results: We identified 4 unbalanced rearrangements: one del 18pter/amp 5pter, one del 9pter, one 15q11q13 microdeletion, and one 22q11 microdeletion with atypical presentation. After genetic counseling, two of the pregnancies were terminated.

Conclusion: MLPA analysis was able to detect 6.5% of clinically-significant cryptic rearrangements. This prospective study highlights that MLPA screening of fetuses with ultrasound abnormalities in the prenatal period is technically feasible and relevant for diagnosis and prognosis.

Keywords: MLPA; prenatal diagnosis; ultrasound malformation; chromosomal cryptic imbalances; molecular cytogenetics.

INTRODUCTION

Congenital malformations are a major cause of morbidity and mortality in newborn infants, and are diagnosed in about 3% of the population. Most major malformations are detected by ultrasound in the prenatal period. Standard karyotype obtained by chorionic villus sampling or amniocentesis reveals chromosomal aberrations in 18-35% of affected fetuses.[1, 2] To increase this detection rate, high-resolution methods based on FISH, comparative genomic hybridization (metaphase spread or array-CGH) and quantitative multiplex PCR techniques such as MLPA (multiplex ligation probe amplification) or QMPSF (quantitative multiplex PCR of short fragments) have been applied but their use in prenatal diagnosis remains limited.

Telomere-specific FISH has already been shown to be a useful tool for detecting deletions and duplications in fetuses with major malformations.[3, 4] More recently, array-CGH analysis of fetal samples for the detection of small-copy-number DNA changes has been established as feasible.[5-8], but application in routine prenatal diagnosis needs further careful evaluation due to the detection of copy number polymorphism of unknown clinical significance.

MLPA is a rapid, cost-effective method for screening for cryptic unbalanced subtelomeric rearrangements and interstitial imbalances located in regions associated with mental retardation (MRS-MLPA). To date, only one study has combined MRS-MLPA with subtelomeric MLPA, which improved the imbalance detection rate from 5.8% to 10.1% in children with mental retardation.[9]

This study screened 61 fetuses with normal karyotype and ultrasound abnormalities suggestive of chromosomal aberration using subtelomeric MLPA and MRS-MLPA. To our knowledge, this is the first report of subtelomeric MLPA combined with MRS-MLPA used as a prenatal prospective screening method in the diagnostic evaluation of fetuses with ultrasound abnormalities.

MATERIALS AND METHODS

Patients

Sixty-one fetuses with one or more malformations suggestive of chromosomal abnormalities were included in our prospective study over the period January 2007 to January 2009 (Table 1). For these 61 fetuses, a 400-band resolution karyotype was performed from amniotic fluid or chorionic villi, and was found to be normal. In cases of cardiac abnormalities, a 22q11.2 FISH was performed. Appropriate informed consent was obtained from all patients, and the study was performed in full compliance with Declaration of Helsinki principles.

MLPA

DNA was isolated from cultured chorionic villi or amniotic fluid cells using the NucleoSpin Blood kit (MACHEREY-NAGEL) following the manufacturer's instructions. MLPA was performed using SALSA P036 and P070 probe sets for subtelomere analysis, followed by MRS-MLPA using a SALSA P245 probe set when negative (MRC-Holland, Amsterdam, The Netherlands). One hundred-and-twenty nanograms of DNA were used in the MLPA protocol according to the manufacturer's instructions. Reactions were performed on a TProfessional thermocycler (Biometra). Three microliters of PCR products were analyzed by capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems), and quantitative data were extracted using ABI Prism GeneScan Analysis and Genetyper software (Applied Biosystems). MLPA data analysis was performed using Microsoft Excel. Abnormal results were defined by a ratio above 1.3 for gain and below 0.7 for loss of genetic material.

FISH

Abnormal findings obtained by MLPA were validated by FISH on metaphase chromosomes according to standard procedures (Cytocell Technology or Vysis, Inc, Downers Grove, IL) using probes located in the suspected regions plus control probes. Peripheral blood lymphocytes from the parents were analyzed by FISH when a fetus tested was positive for rearrangement.

RESULTS

We detected cryptic imbalances in four of the 61 fetuses (6.5%) explored by MLPA. Two subtelomeric rearrangements and two pericentromeric microdeletions were identified. The clinical and cytogenetic data for these four cases are given below.

Patient A. The 24-year-old mother of this fetus had one healthy child and one miscarriage. Family history was nonsignificant. At 13 weeks of gestation, nuchal translucency was measured at 5.3 mm. Prenatal cytogenetic analysis of chorionic villi revealed a normal karyotype: 46,XX. An ultrasound performed at 18 weeks of gestation showed a suspected cardiac defect, and the patient underwent fetal echocardiography at 19 weeks of gestation which revealed a complex cardiac defect with aortic valve agenesia and left ventricular dysfunction associated with poor prognosis. Further cytogenetic investigations were then conducted on the cryoconserved cells. Subtelomeric MLPA analysis revealed a loss of 18qter and a gain of 5pter (figure 1a), which were FISH confirmed as an unbalanced translocation product (see figure 2a, supplemental digital content). Karyotype and FISH analysis on metaphase spreads of the parents showed no aberrations. After genetic counseling, pregnancy was terminated at 20 weeks of gestation, in accordance with French legislation. Autopsy revealed severe maceration and partial liquefaction of the intra-abdominal organs, confirmed the complex heart defect, and revealed a single umbilical artery and bilateral pulmonary hypoplasia.

Patient B. The 26-year-old mother of this fetus was pregnant with her second child. First-trimester ultrasound scan measured nuchal translucency thickness at 1.4 mm, and maternal serum screening gave a Down syndrome risk estimate of 1:840. Family history was nonsignificant. At 34 weeks of gestation, ultrasound examination revealed intrauterine growth retardation and hypospadias, prompting to send amniotic fluid from fetus C to the laboratory for cytogenetic analysis. The karyotype, 46,XY, was normal, and FISH testing for 4p16.3 deletion (Wolf-Hirschhorn, OMIM194190) was negative. Subtelomeric MLPA analysis revealed a loss of 9pter (figure 1b), and the deletion was confirmed by FISH (see figure 2b, supplemental digital content). Karyotype and FISH analysis on metaphase spreads of the parents showed no aberrations. Following counseling, and in accordance with French legislation, the parents decided to terminate the pregnancy at 38 weeks of gestation due to the high risk of mental retardation. The post-mortem clinical assessment confirmed the

hypospadias and showed a facial dysmorphism with upslanted palpebral fissures, microretrognatia, short neck and widely-spaced nipples.

Patient C. The patient was a 30-year-old gravida 2 para 1 with an unremarkable personal history. First-trimester ultrasound scan measured nuchal translucency thickness at 1.4 mm. Maternal serum screening gave a Down syndrome (OMIM190685) risk estimate of 1:1388. Ultrasound examination at 33 weeks of gestation revealed polyhydramnios associated with diminished fetal movements. Amniocentesis was performed for fetal karyotype and molecular investigations of Steinert's disease (OMIM160900). Both the Steinert's disease test and the fetal karyotype (46,XX) were normal. At 36 weeks of gestation, the subtelomeric MLPA detected a 15q11q13 microdeletion (figure 1c) which was also identified by MRS-MLPA (data not shown). It is important to note that the P036 and P070 probe sets did not explore the p-arm of the 5 acrocentric chromosomes but the q-arm close to the centromere. The microdeletion was then validated by FISH (see figure 2c, supplemental digital content). The parents decided to continue the pregnancy, and delivery occurred at 39 weeks of gestation. The post-natal assessment showed hypotonia, poor sucking (requiring gavage feeding), small hands and feet with mild edema at the dorsum of feet, ogival palate, and facial dysmorphism typical of Prader-Willi syndrome (PWS, OMIM176270). Weight was 3080 g, length 50 cm, and occipital frontal circumference 36 cm.

Patient D. At 25 weeks of gestation, the 30-year-old primagravida mother underwent amniocentesis due to unilateral dysplasic multicystic kidney and bilateral club feet. First-trimester nuchal translucency thickness was measured at 1.6 mm and maternal serum screening gave a low Down syndrome risk estimate of 1:2899. Family history was nonsignificant, and parental renal ultrasounds were normal. The fetal karyotype, 46,XY, was normal. At 32 weeks of gestation, significant polyhydramnios was detected, and further cytogenetic investigations were performed on the cryoconserved cells. At 35 weeks of gestation, MRS-MLPA detected a 22q11.2 microdeletion (figure 1d) which was confirmed by FISH (see figure 2d, supplemental digital content). The parents refused to undergo their own karyotype testing and, after genetic counseling, decided to continue the pregnancy. The post-natal assessment at term confirmed the fetal abnormalities, and additional malformations were diagnosed. These included perimembranous interventricular communication, laryngeal stridor, and severe gastroesophageal reflux. Weight was 3540 g, length 50.5 cm, and occipital frontal circumference 36 cm.

DISCUSSION

The objective of this study was to evaluate the technical feasibility and utility of performing MLPA during the prenatal period. To our knowledge, this is the first study to assess MRS-MLPA combined with subtelomeric MLPA in the prenatal diagnostic screening of fetuses with abnormal ultrasound of unknown etiology.

We detected 4 submicroscopic rearrangements using three different MLPA probe sets in the 61 fetuses referred to our laboratory for abnormal ultrasound. These imbalances were confirmed by FISH on metaphase spreads.

Two subtelomeric rearrangements with clinical significance (del 18pter/amp 5pter and del 9pter) were detected (3.3%). These data are consistent with the 3-7% frequency of subtelomeric rearrangements detected by FISH, MLPA, quantitative PCR and array-CGH in patients with congenital defects and/or mental retardation.[4,10-14] However, there are few reports concerning the detection of such imbalances in the prenatal period. Souter et *al* reported two prenatal cases of multiple fetal anomalies associated with subtle subtelomeric rearrangements detected by multi-subtelomere FISH.[3] Gignac et *al* also screened 48 polymalformed fetuses with multi-subtelomere FISH, and identified two clinically significant subtelomeric rearrangements (4%).[4] More recently, Faas et *al* reported the detection of subtelomeric imbalance by FISH and MLPA in three fetuses presenting ultrasound abnormalities. Their MLPA analysis was performed retrospectively in the post-natal period, and therefore had no influence on prenatal decision-making.[15]

We also detected two interstitial deletions (3.3%). Both subtelomeric MLPA and MRS-MLPA detected a 15q11q13 microdeletion associated with polyhydramnios and hypotonia. Recently, Bigi et *al* published the first report to describe a possible fetal phenotype in PWS which could be recognizable after 30 weeks of gestation.[16] They claimed that a particular position of hands and feet combined with diminished fetal movements and polyhydramnios is suggestive of PWS.[16] However, fetus C did not present this kind of malposition of the extremities. Dudley & Muscatelly described a genotype-dependent variation in the obstetric characteristics of PWS, i.e. deletion or maternal uniparental disomy, making the fetal phenotype difficult to define.[17] MRS-MLPA also detected a 22q11.2 microdeletion with atypical presentation. Renal dysplasia is frequently associated with 22q11.2 microdeletion, and there are multiple reports of polyhydramnios and club feet in this syndrome.[18,19] However, to our knowledge, the prenatal association of unilateral dysplasic multicystic kidney, bilateral club feet and polyhydramnios identified in fetus D has never been reported in 22q11.2 microdeletion syndrome. Taken together, these two cases suggest

that MRS-MLPA may usefully detect microdeletion syndromes with atypical or incomplete prenatal presentation.

Array-CGH analysis has also been successfully applied for detection of chromosomal imbalances on fetal samples.[5,7,8] However, arrays which cover the whole genome at high resolution have identified a large number of copy number polymorphisms within the normal population.[20] Thus, high-resolution array-CGH could detect copy number variations not directly associated with abnormal phenotype, and thus generate data of unknown significance.[21] Hence, if array-CGH is to be used for prenatal diagnosis, the format of the array and the validation of results warrant careful consideration.[6] Our results suggest that MLPA-based screening, particularly MRS-MLPA combined with subtelomeric MLPA, on fetuses with abnormal ultrasound would be a more appropriate first-line routine diagnostic procedure when the karyotype is normal and echographic signs strongly suggest chromosomal abnormalities. Furthermore, MLPA is cheaper and less time-intensive than array-CGH, and it can analyze several patients in one run. MLPA-targeted genes located very close to the telomere have a higher chance of presenting a polymorphic copy number. However, the detection of such polymorphisms can be overcome by simultaneously using subtelomeric SALSA P036 and P070 probe sets. In our experience, positive results found with both kits are more likely to be non-inherited imbalances causing the abnormal phenotype. Normal MLPA screening could then be followed by dedicated array-CGH for prenatal diagnosis for cases with high clinical suspicion of chromosome abnormality.

In conclusion, we demonstrate the technical feasibility of MLPA on prenatal samples and describe for the first time the utility of subtelomeric MLPA and MRS-MLPA for prenatal diagnosis of cryptic chromosomal imbalances. Our results suggest that these cryptic rearrangements may be an under-recognized cause of multiple fetal malformations, and that MLPA may be useful as a routine diagnostic procedure. The prenatal applicability of this cost-effective technique gives a more precise prognosis, and influences the perinatal management and care.

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Figure legend

Figure 1: Gene dosage ratios for subtelomeric MLPA and MRS-MLPA. (a) Dosage ratio of SALSA P036 (left) and P070 (right) probe sets for fetus A showing an amplification of the 5p telomere and a deletion of the 18q telomere. (b) Dosage ratio of SALSA P036 (left) and P070 (right) probe sets for fetus B showing a deletion of the 9p telomere. (c) Dosage ratio of SALSA P036 (left) and P070 (right) probe sets for fetus C showing a deletion of the 15q11.2 region. (d) Dosage ratio of SALSA P245 probe sets for fetus D showing deletion of three probes targeting the 22q11.21 region.

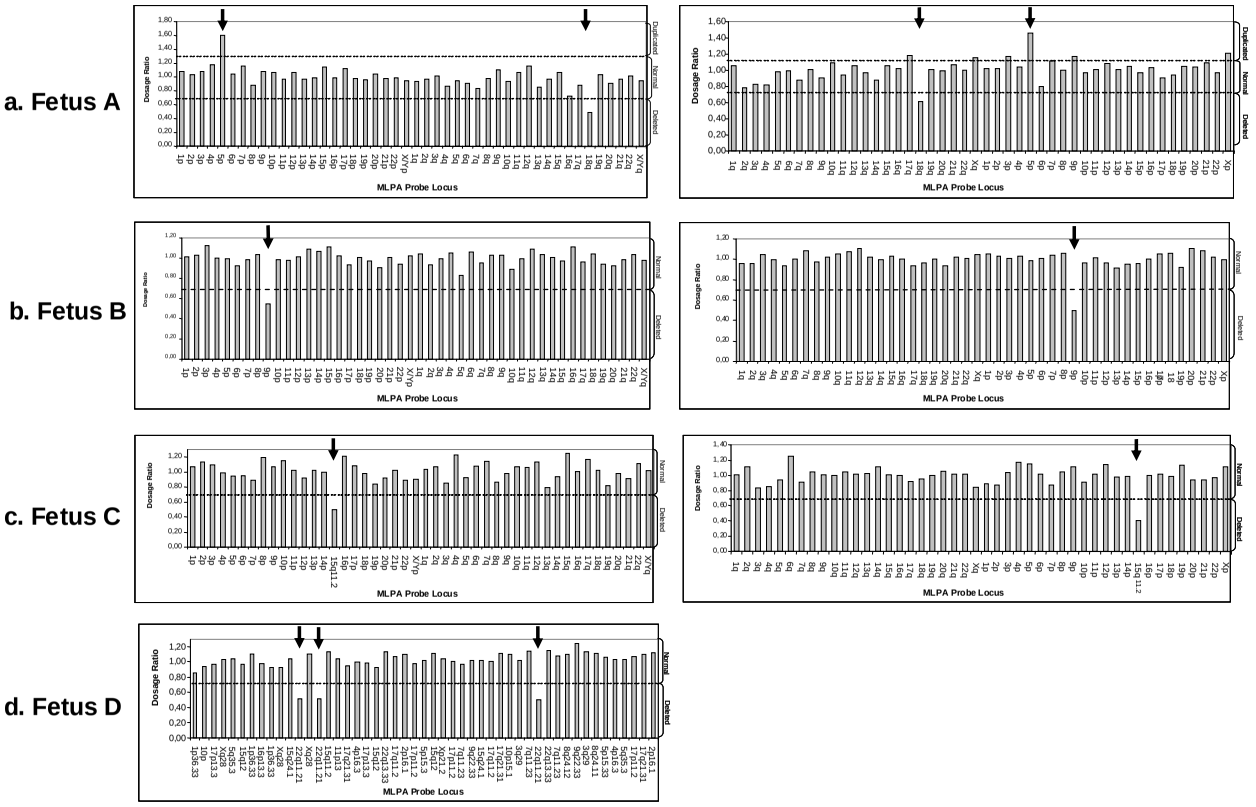
Table 1. Summary of clinical ultrasound abnormalities

Ultrasound abnormalities	Number of cases
Isolated defect	**36**
- cerebral	12
- cardiac	5
- skeletal*	4
- genital	2
- digestive	2
- diaphragmatic hernia	6
- facial dysmorphism	5
Isolated IUGR	**10**
IUGR with malformation	**5**
- genital	2
- cardiac	1
- cerebral	1
- rachischisis	1
Multiple malformations	**5**
- cerebral and skeletal[a]	2
- cardiac and cerebral	1
- skeletal* and renal	1
- cardiac and skeletal*	1
Abnormal amniotic fluid volume	**4**
Other	**1**

IUGR = Intrauterine growth restriction (below 3rd percentile)

[a] limb malformations

Figure 1



List of Supplemental Digital Content

Figure 2 Supplemental Digital Content 1, Figure that shows FISH analysis for each MLPA results . ppt

Abstract

Purpose: Congenital malformations are a major cause of morbidity and mortality in newborn infants, and genomic imbalances are a significant component of their etiology. The aim of this study was to evaluate the ability of prenatal MLPA (Multiplex Ligation Probe Amplification) screening to detect cryptic chromosomal imbalances in fetuses with ultrasound abnormalities of unknown etiology.

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INTRODUCTION

Congenital malformations are a major cause of morbidity and mortality in newborn infants, and are diagnosed in about 3% of the population. Most major malformations are detected by ultrasound in the prenatal period. Standard karyotype obtained by chorionic villus sampling or amniocentesis reveals chromosomal aberrations in 18-35% of affected fetuses.[1, 2] To increase this detection rate, high-resolution methods based on FISH, comparative genomic hybridization (metaphase spread or array-CGH) and quantitative multiplex PCR techniques such as MLPA (multiplex ligation probe amplification) or QMPSF (quantitative multiplex PCR of short fragments) have been applied but their use in prenatal diagnosis remains limited.

Telomere-specific FISH has already been shown to be a useful tool for detecting deletions and duplications in fetuses with major malformations.[3, 4] More recently, array-CGH analysis of fetal samples for the detection of small-copy-number DNA changes has been established as feasible.[5-8], but application in routine prenatal diagnosis needs further careful evaluation due to the detection of copy number polymorphism of unknown clinical significance.

MLPA is a rapid, cost-effective method for screening for cryptic unbalanced subtelomeric rearrangements and interstitial imbalances located in regions associated with mental retardation (MRS-MLPA). To date, only one study has combined MRS-MLPA with subtelomeric MLPA, which improved the imbalance detection rate from 5.8% to 10.1% in children with mental retardation.[9]

This study screened 61 fetuses with normal karyotype and ultrasound abnormalities suggestive of chromosomal aberration using subtelomeric MLPA and MRS-MLPA. To our knowledge, this is the first report of subtelomeric MLPA combined with MRS-MLPA used as a prenatal prospective screening method in the diagnostic evaluation of fetuses with ultrasound abnormalities.

MATERIALS AND METHODS

Patients

Sixty-one fetuses with one or more malformations suggestive of chromosomal abnormalities were included in our prospective study over the period January 2007 to January 2009 (Table 1). For these 61 fetuses, a 400-band resolution karyotype was performed from amniotic fluid or chorionic villi, and was found to be normal. In cases of cardiac abnormalities, a 22q11.2 FISH was performed. Appropriate informed consent was obtained from all patients, and the study was performed in full compliance with Declaration of Helsinki principles.

MLPA

DNA was isolated from cultured chorionic villi or amniotic fluid cells using the NucleoSpin Blood kit (MACHEREY-NAGEL) following the manufacturer's instructions. MLPA was performed using SALSA P036 and P070 probe sets for subtelomere analysis, followed by MRS-MLPA using a SALSA P245 probe set when negative (MRC-Holland, Amsterdam, The Netherlands). One hundred-and-twenty nanograms of DNA were used in the MLPA protocol according to the manufacturer's instructions. Reactions were performed on a TProfessional thermocycler (Biometra). Three microliters of PCR products were analyzed by capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems), and quantitative data were extracted using ABI Prism GeneScan Analysis and Genetyper software (Applied Biosystems). MLPA data analysis was performed using Microsoft Excel. Abnormal results were defined by a ratio above 1.3 for gain and below 0.7 for loss of genetic material.

FISH

Abnormal findings obtained by MLPA were validated by FISH on metaphase chromosomes according to standard procedures (Cytocell Technology or Vysis, Inc, Downers Grove, IL) using probes located in the suspected regions plus control probes. Peripheral blood lymphocytes from the parents were analyzed by FISH when a fetus tested was positive for rearrangement.

RESULTS

We detected cryptic imbalances in four of the 61 fetuses (6.5%) explored by MLPA. Two subtelomeric rearrangements and two pericentromeric microdeletions were identified. The clinical and cytogenetic data for these four cases are given below.

Patient A. The 24-year-old mother of this fetus had one healthy child and one miscarriage. Family history was nonsignificant. At 13 weeks of gestation, nuchal translucency was measured at 5.3 mm. Prenatal cytogenetic analysis of chorionic villi revealed a normal karyotype: 46,XX. An ultrasound performed at 18 weeks of gestation showed a suspected cardiac defect, and the patient underwent fetal echocardiography at 19 weeks of gestation which revealed a complex cardiac defect with aortic valve agenesia and left ventricular dysfunction associated with poor prognosis. Further cytogenetic investigations were then conducted on the cryoconserved cells. Subtelomeric MLPA analysis revealed a loss of 18qter and a gain of 5pter (figure 1a), which were FISH confirmed as an unbalanced translocation product (see figure 2a, supplemental digital content). Karyotype and FISH analysis on metaphase spreads of the parents showed no aberrations. After genetic counseling, pregnancy was terminated at 20 weeks of gestation, in accordance with French legislation. Autopsy revealed severe maceration and partial liquefaction of the intra-abdominal organs, confirmed the complex heart defect, and revealed a single umbilical artery and bilateral pulmonary hypoplasia.

Patient B. The 26-year-old mother of this fetus was pregnant with her second child. First-trimester ultrasound scan measured nuchal translucency thickness at 1.4 mm, and maternal serum screening gave a Down syndrome risk estimate of 1:840. Family history was nonsignificant. At 34 weeks of gestation, ultrasound examination revealed intrauterine growth retardation and hypospadias, prompting to send amniotic fluid from fetus C to the laboratory for cytogenetic analysis. The karyotype, 46,XY, was normal, and FISH testing for 4p16.3 deletion (Wolf-Hirschhorn, OMIM194190) was negative. Subtelomeric MLPA analysis revealed a loss of 9pter (figure 1b), and the deletion was confirmed by FISH (see figure 2b, supplemental digital content). Karyotype and FISH analysis on metaphase spreads of the parents showed no aberrations. Following counseling, and in accordance with French legislation, the parents decided to terminate the pregnancy at 38 weeks of gestation due to the high risk of mental retardation. The post-mortem clinical assessment confirmed the

hypospadias and showed a facial dysmorphism with upslanted palpebral fissures, microretrognatia, short neck and widely-spaced nipples.

Patient C. The patient was a 30-year-old gravida 2 para 1 with an unremarkable personal history. First-trimester ultrasound scan measured nuchal translucency thickness at 1.4 mm. Maternal serum screening gave a Down syndrome (OMIM190685) risk estimate of 1:1388. Ultrasound examination at 33 weeks of gestation revealed polyhydramnios associated with diminished fetal movements. Amniocentesis was performed for fetal karyotype and molecular investigations of Steinert's disease (OMIM160900). Both the Steinert's disease test and the fetal karyotype (46,XX) were normal. At 36 weeks of gestation, the subtelomeric MLPA detected a 15q11q13 microdeletion (figure 1c) which was also identified by MRS-MLPA (data not shown). It is important to note that the P036 and P070 probe sets did not explore the p-arm of the 5 acrocentric chromosomes but the q-arm close to the centromere. The microdeletion was then validated by FISH (see figure 2c, supplemental digital content). The parents decided to continue the pregnancy, and delivery occurred at 39 weeks of gestation. The post-natal assessment showed hypotonia, poor sucking (requiring gavage feeding), small hands and feet with mild edema at the dorsum of feet, ogival palate, and facial dysmorphism typical of Prader-Willi syndrome (PWS, OMIM176270). Weight was 3080 g, length 50 cm, and occipital frontal circumference 36 cm.

Patient D. At 25 weeks of gestation, the 30-year-old primagravida mother underwent amniocentesis due to unilateral dysplasic multicystic kidney and bilateral club feet. First-trimester nuchal translucency thickness was measured at 1.6 mm and maternal serum screening gave a low Down syndrome risk estimate of 1:2899. Family history was nonsignificant, and parental renal ultrasounds were normal. The fetal karyotype, 46,XY, was normal. At 32 weeks of gestation, significant polyhydramnios was detected, and further cytogenetic investigations were performed on the cryoconserved cells. At 35 weeks of gestation, MRS-MLPA detected a 22q11.2 microdeletion (figure 1d) which was confirmed by FISH (see figure 2d, supplemental digital content). The parents refused to undergo their own karyotype testing and, after genetic counseling, decided to continue the pregnancy. The post-natal assessment at term confirmed the fetal abnormalities, and additional malformations were diagnosed. These included perimembranous interventricular communication, laryngeal stridor, and severe gastroesophageal reflux. Weight was 3540 g, length 50.5 cm, and occipital frontal circumference 36 cm.

DISCUSSION

The objective of this study was to evaluate the technical feasibility and utility of performing MLPA during the prenatal period. To our knowledge, this is the first study to assess MRS-MLPA combined with subtelomeric MLPA in the prenatal diagnostic screening of fetuses with abnormal ultrasound of unknown etiology.

We detected 4 submicroscopic rearrangements using three different MLPA probe sets in the 61 fetuses referred to our laboratory for abnormal ultrasound. These imbalances were confirmed by FISH on metaphase spreads.

Two subtelomeric rearrangements with clinical significance (del 18pter/amp 5pter and del 9pter) were detected (3.3%). These data are consistent with the 3-7% frequency of subtelomeric rearrangements detected by FISH, MLPA, quantitative PCR and array-CGH in patients with congenital defects and/or mental retardation.[4,10-14] However, there are few reports concerning the detection of such imbalances in the prenatal period. Souter et *al* reported two prenatal cases of multiple fetal anomalies associated with subtle subtelomeric rearrangements detected by multi-subtelomere FISH.[3] Gignac et *al* also screened 48 polymalformed fetuses with multi-subtelomere FISH, and identified two clinically significant subtelomeric rearrangements (4%).[4] More recently, Faas et *al* reported the detection of subtelomeric imbalance by FISH and MLPA in three fetuses presenting ultrasound abnormalities. Their MLPA analysis was performed retrospectively in the post-natal period, and therefore had no influence on prenatal decision-making.[15]

We also detected two interstitial deletions (3.3%). Both subtelomeric MLPA and MRS-MLPA detected a 15q11q13 microdeletion associated with polyhydramnios and hypotonia. Recently, Bigi et *al* published the first report to describe a possible fetal phenotype in PWS which could be recognizable after 30 weeks of gestation.[16] They claimed that a particular position of hands and feet combined with diminished fetal movements and polyhydramnios is suggestive of PWS.[16] However, fetus C did not present this kind of malposition of the extremities. Dudley & Muscatelly described a genotype-dependent variation in the obstetric characteristics of PWS, i.e. deletion or maternal uniparental disomy, making the fetal phenotype difficult to define.[17] MRS-MLPA also detected a 22q11.2 microdeletion with atypical presentation. Renal dysplasia is frequently associated with 22q11.2 microdeletion, and there are multiple reports of polyhydramnios and club feet in this syndrome.[18,19] However, to our knowledge, the prenatal association of unilateral dysplasic multicystic kidney, bilateral club feet and polyhydramnios identified in fetus D has never been reported in 22q11.2 microdeletion syndrome. Taken together, these two cases suggest

that MRS-MLPA may usefully detect microdeletion syndromes with atypical or incomplete prenatal presentation.

Array-CGH analysis has also been successfully applied for detection of chromosomal imbalances on fetal samples.[5,7,8] However, arrays which cover the whole genome at high resolution have identified a large number of copy number polymorphisms within the normal population.[20] Thus, high-resolution array-CGH could detect copy number variations not directly associated with abnormal phenotype, and thus generate data of unknown significance.[21] Hence, if array-CGH is to be used for prenatal diagnosis, the format of the array and the validation of results warrant careful consideration.[6] Our results suggest that MLPA-based screening, particularly MRS-MLPA combined with subtelomeric MLPA, on fetuses with abnormal ultrasound would be a more appropriate first-line routine diagnostic procedure when the karyotype is normal and echographic signs strongly suggest chromosomal abnormalities. Furthermore, MLPA is cheaper and less time-intensive than array-CGH, and it can analyze several patients in one run. MLPA-targeted genes located very close to the telomere have a higher chance of presenting a polymorphic copy number. However, the detection of such polymorphisms can be overcome by simultaneously using subtelomeric SALSA P036 and P070 probe sets. In our experience, positive results found with both kits are more likely to be non-inherited imbalances causing the abnormal phenotype. Normal MLPA screening could then be followed by dedicated array-CGH for prenatal diagnosis for cases with high clinical suspicion of chromosome abnormality.

In conclusion, we demonstrate the technical feasibility of MLPA on prenatal samples and describe for the first time the utility of subtelomeric MLPA and MRS-MLPA for prenatal diagnosis of cryptic chromosomal imbalances. Our results suggest that these cryptic rearrangements may be an under-recognized cause of multiple fetal malformations, and that MLPA may be useful as a routine diagnostic procedure. The prenatal applicability of this cost-effective technique gives a more precise prognosis, and influences the perinatal management and care.

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Figure legend

Figure 1: Gene dosage ratios for subtelomeric MLPA and MRS-MLPA. (a) Dosage ratio of SALSA P036 (left) and P070 (right) probe sets for fetus A showing an amplification of the 5p telomere and a deletion of the 18q telomere. (b) Dosage ratio of SALSA P036 (left) and P070 (right) probe sets for fetus B showing a deletion of the 9p telomere. (c) Dosage ratio of SALSA P036 (left) and P070 (right) probe sets for fetus C showing a deletion of the 15q11.2 region. (d) Dosage ratio of SALSA P245 probe sets for fetus D showing deletion of three probes targeting the 22q11.21 region.

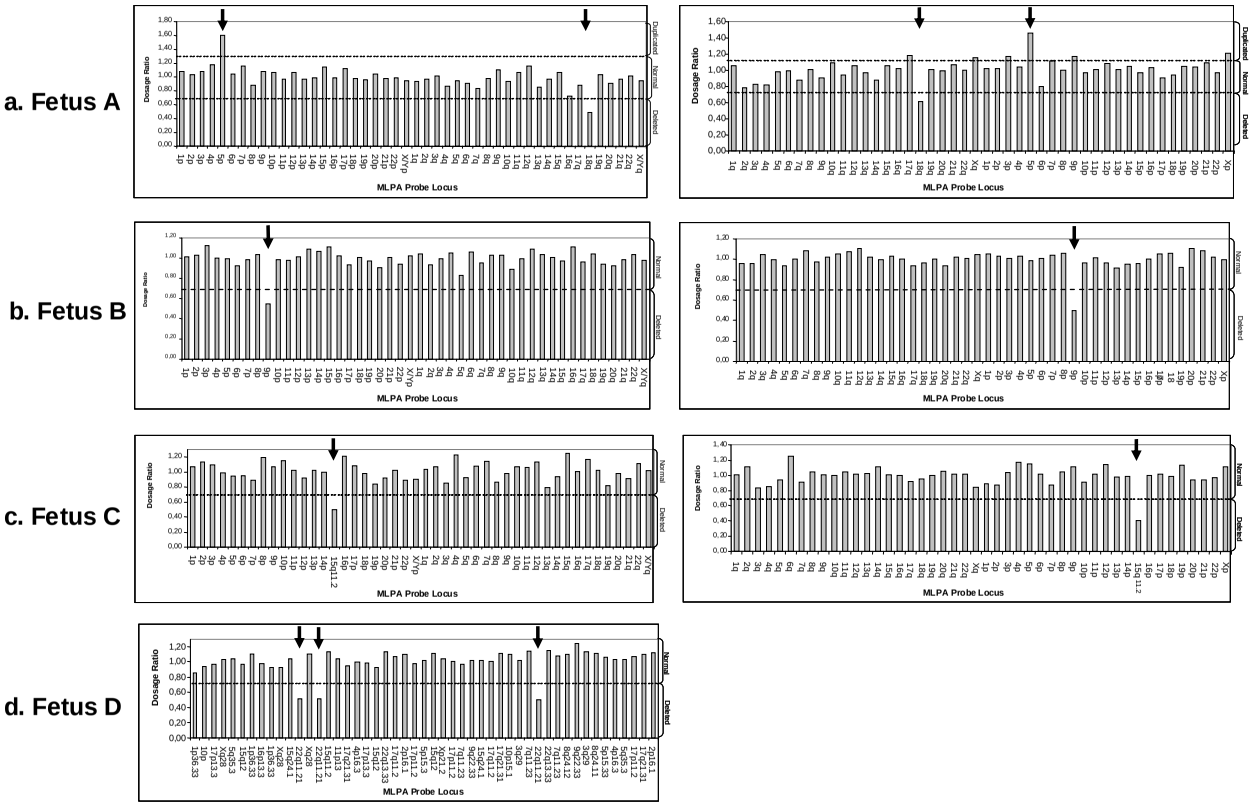
Table 1. Summary of clinical ultrasound abnormalities

Ultrasound abnormalities	Number of cases
Isolated defect	**36**
- cerebral	12
- cardiac	5
- skeletal*	4
- genital	2
- digestive	2
- diaphragmatic hernia	6
- facial dysmorphism	5
Isolated IUGR	**10**
IUGR with malformation	**5**
- genital	2
- cardiac	1
- cerebral	1
- rachischisis	1
Multiple malformations	**5**
- cerebral and skeletal[a]	2
- cardiac and cerebral	1
- skeletal* and renal	1
- cardiac and skeletal*	1
Abnormal amniotic fluid volume	**4**
Other	**1**

IUGR = Intrauterine growth restriction (below 3rd percentile)

[a] limb malformations

Figure 1



List of Supplemental Digital Content

Figure 2 Supplemental Digital Content 1, Figure that shows FISH analysis for each MLPA results . ppt

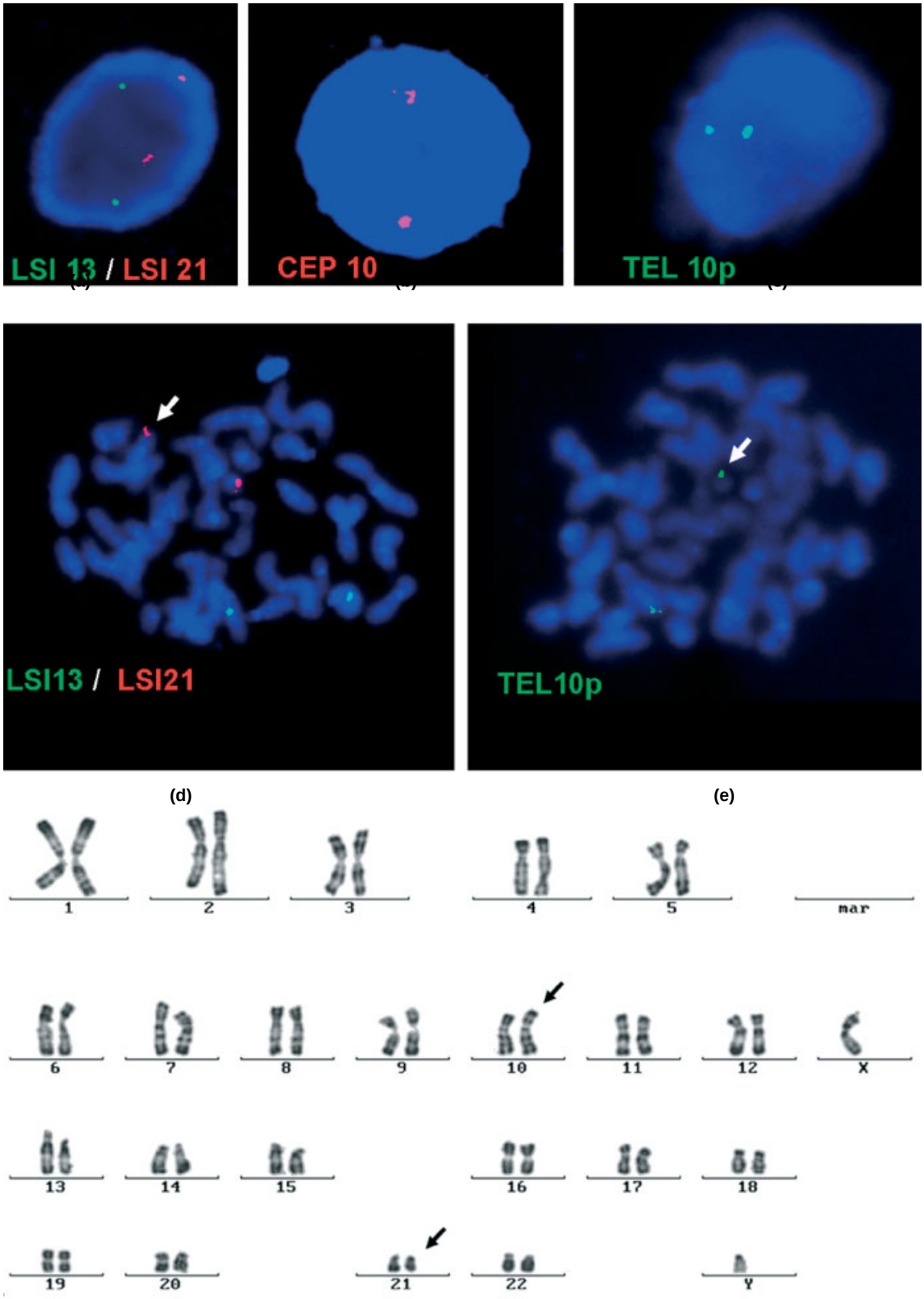


Figure 1—(a–c) FISH on interphasic nuclei from CV direct preparations. (a) two signals for LSI 13 (green) and LSI 21 (red) probes; (b) two signals for CEP 10 (red); (c) two signals for TEL 10p probe (green). The fetus is balanced for these regions. (d,e) FISH on spontaneous metaphases. (d) One of the two signals for LSI 21 probe (red) is carried by a group C chromosome (arrow); (e) one of the two signals for TEL 10p probe (green) is carried by a group G chromosome (arrow). The fetus carries the maternal translocation 10;21; (f) the RGH karyotype from cultured CV cells is 46,XY,t(10;21)(p12;q11)mat

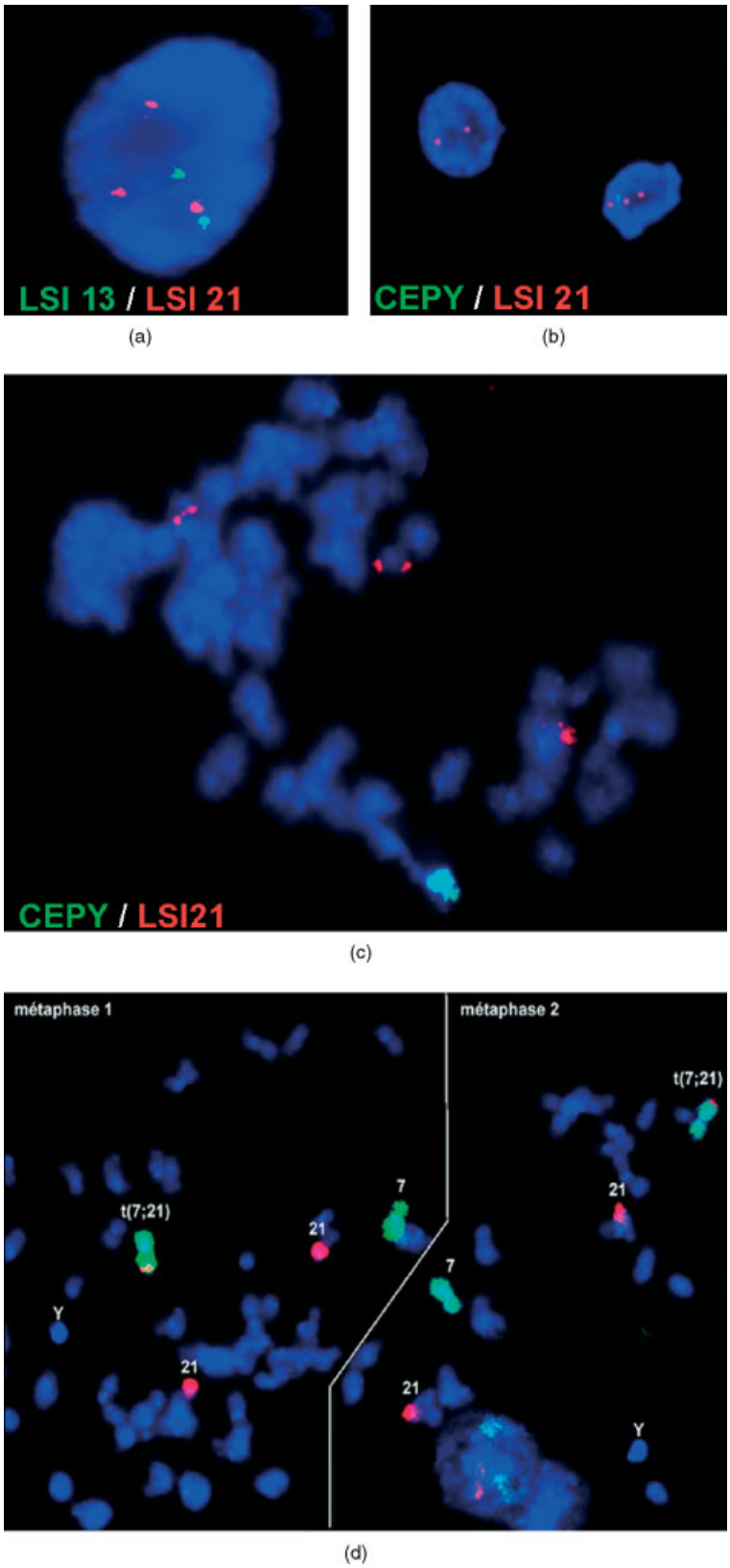


Figure 2—(a) FISH on interphasic nuclei from CV direct preparations. Three signals for LSI 21 probe (red). (b,c) FISH after CV cell culture; (b) three signals for LSI 21 probe (red) on a nucleus CEP Y +; (c) three signals for LSI 21 probe (red) on a metaphase CEP Y +: the fetal trisomy 21 is confirmed. (d) WCP 7 (green) and WCP 21 (red) on metaphases from fetal cells after culture: the fetus carried one chromosome 7, two chromosomes 21 and the derivative 7 of the maternal translocation 7;21. The fetal karyotype is finally established as follows: 46,XY,der(7),t(7;21)(q35;q22)mat

Finally, in the 4;17 translocation, FISH with TEL 4p, TEL 4q and TEL 17q probes showed two copies of each probe (Figure 4a and b), and a single direct metaphase suggested that the fetus had a balanced translocation (Figure 4c). This was also confirmed by the karyotype.

DISCUSSION

The present study shows that FISH analysis of CVS is extremely accurate in detecting numerical aberrations for chromosomes 13, 18, 21, X and Y, and is able to rapidly exclude an unbalanced karyotype in the fetus in case of parental translocation.

Because the main indication for early CVS was abnormal ultrasound, a very high rate of chromosomal aberration was discovered.

The Vysis prenatal detection kit (AneuVysion™) detected all cases of fetal trisomy 13, 18 and 21 and monosomy X. The pregnancies were interrupted before the 12th week of gestation, and the results were subsequently confirmed on fetal material. In their 2-year multicentric retrospective study, Tepperberg and colleagues (2001) reported an extremely high concordance rate (99.8%) between FISH and standard cytogenetics for the specific abnormalities that the AneuVysion™ assay is designed to detect. In our study, no false results were encountered. Although our sample size was relatively small, the probe signals were of consistently high quality in both normal and aneuploid cases.

Our results also demonstrated the ability of FISH analysis to rapidly identify the balanced or unbalanced status of a fetus when one of the parents is a carrier of balanced translocation. With a three-probes set—two probes for one chromosome on either side of the breakpoint and one probe for the other chromosome involved—each possible abnormal segregant outcome can be distinguished. This approach gives a rapid preliminary result that can significantly reduce parental anxiety, which is common in couples carrying a balanced translocation, particulary if ascertained through the birth of a chromosomally abnormal child or a fetal demise. When abnormal results are found, this rapid prenatal diagnosis allows early termination of pregnancy by simple suction curettage.

The only inconsequential restriction of this approach is that FISH analysis cannot distinguish between the

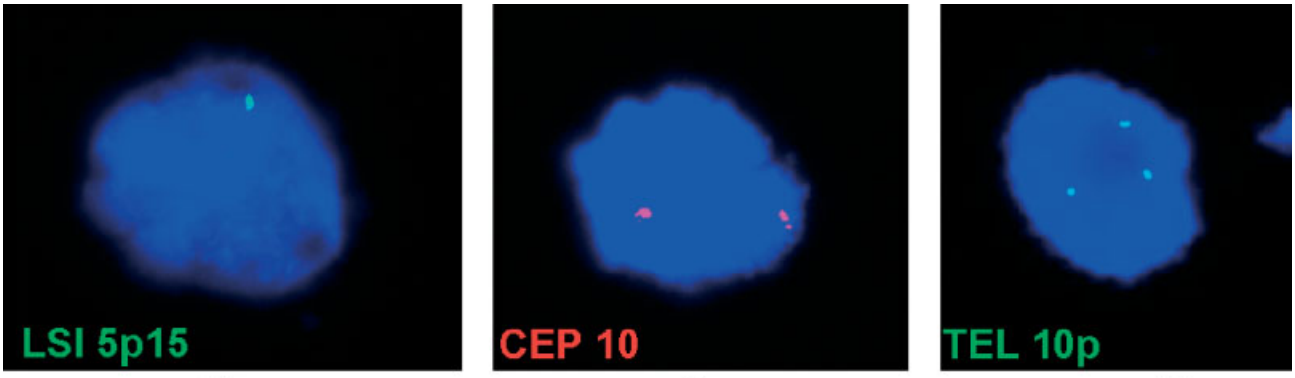


Figure 3—FISH on interphasic nuclei from CV direct preparations. (a) One signal for LSI 5p15 probe (green); (b) two signals for CEP 10 probe (red); (c) three signals for TEL 10p probe (green). The fetus is unbalanced with a partial 10p trisomy and a partial 5p monosomy

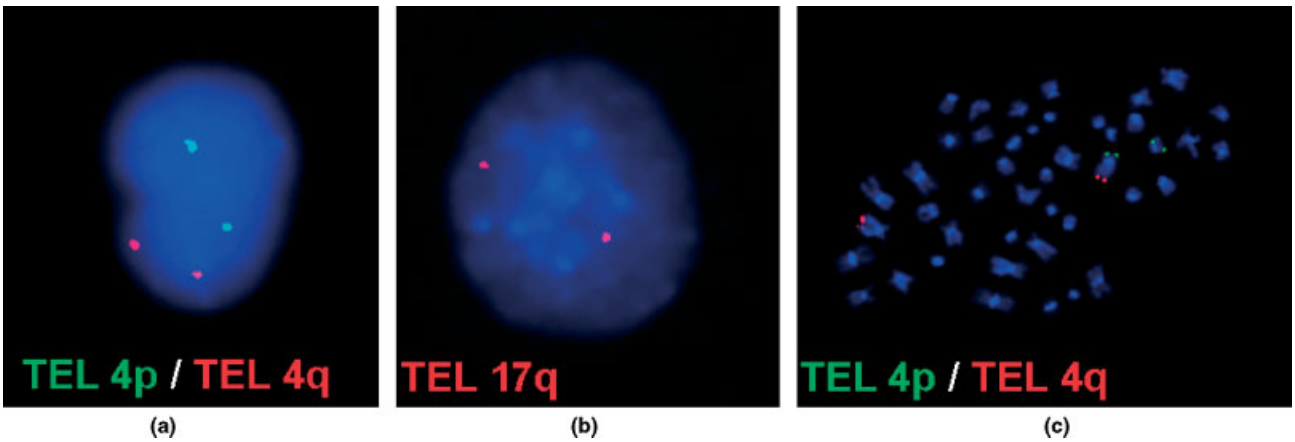


Figure 4—(a,b) FISH on interphasic nuclei from CV direct preparations. (a) Two signals for TEL 4p (green) and TEL 4q (red) probes; (b) two signals for TEL 17q probes (red): the fetus is balanced for these loci. (c) FISH on a direct metaphase with TEL 4p (green signal) and TEL 4q (red signal) probes: the fetus carries the maternal translocation

balanced alternate segregant and the normal alternate segregant when the sample does not exhibit spontaneous metaphases. Hence, karyotype analysis of cultured cells is required in addition to the interphase analysis. In view of the high recurrence of risk in some of these families, preimplantation diagnosis is now considered.

FISH analysis was always followed by karyotypic analysis of mesenchymal cells. Although careful selection of decidual fragments under a dissecting microscope greatly reduces the number of presumed maternal cells present, 2 out of 13 46,XY samples had maternal (XX) cell contamination, which led to discordance between the FISH analysis and standard cytogenetic investigations. In these two cases, the maternal cells, and not the fetal cells, were karyotyped.

Without FISH, two false-negative results would have been obtained. Thus, FISH assay is of considerable benefit because cultures are not needed. With FISH, sample contamination by maternal cells does not cause problems of interpretation because the proportion of maternal cells before culture is rather small. Moreover, FISH analysis provides a more accurate estimate of tested chromosome mosaicism than conventional chromosome analysis. FISH can rapidly analyse more cells in comparison to standard cytogenetics. Reducing maternal cell contamination has been achieved by a combination of thorough dissection of the material, reduced cultivation time and especially improved obstetrical procedure. The level of maternal cell contamination is directly related to the obstetrical procedure and the amount of material. The rate of MCC was consistently lower than 2% when CVS was performed by an experienced operator (Hockstein *et al*., 1998). According to Quilter and colleagues (2001), samples need to be >20 mg for sufficient material to be there for long-term culture and direct preparation. Hence, another advantage of FISH analysis is the small quantity of material required. We successfully performed FISH assays with cell numbers that would have been insufficient for cell culture.

One outstanding question is how to use the interphase FISH results in the clinical management of pregnancies. According to the guidelines of the American College of Medical Genetics (2000), clinical decision management should be based on the presence of two of the three following criteria: positive FISH result, confirmatory karyotype and consistent clinical information. Bryndorf and colleagues (2000) reported that in their experience 72% of terminations of chromosomally abnormal pregnancies were based on FISH and ultrasound results rather than on standard cytogenetic results. The French multicentre study (Luquet *et al*., 2002) recommended the same clinical management as the American College of Medical Genetics (2000). We also think that an abnormal FISH result can be used for clinical decisions when it is associated with a corresponding abnormal ultrasound scan or a corresponding parental balanced translocation.

The one major drawback to FISH analysis is that it can only detect those aberrations for which it has been designed.

Another criticism of the application of FISH in prenatal diagnosis relates to cost versus benefits. This is why indications for FISH, as for CVS, should be rigorously selected. FISH would be clinically justifiable in situations of great parental stress when there is an increased risk of aneuploidy or unbalanced translocation. In low-risk pregnancies, amniocentesis and classic karyotype would be the methods of choice.

The number of cases in the present study was too small from which to draw any comparison with other series, but three salient points did emerge. FISH minimizes the problem of maternal cell contamination. When associated with CVS in the first trimester of high-risk pregnancies, it rapidly provides a diagnosis that helps reduce anxiety and guides the decision to carry out early termination by simple suction curettage, a choice that is more willingly consented to by parents. Finally, the association, FISH–CVS, may be very useful in cases of familial balanced translocation.

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Gene dosage methods as diagnostic tools for the identification of chromosome abnormalities

Méthodes de dosage génique comme outils diagnostic pour l'identification d'anomalies chromosomiques

L. Gouas *, C. Goumy, L. Véronèse, A. Tchirkov, P. Vago

Service de cytogénétique médicale, UFR médecine, histologie embryologie cytogénétique, CHU Clermont-Ferrand, faculté de médecine, université Clermont-1, B.P. 38, 63001 Clermont-Ferrand, France

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Abstract

Cytogenetics is the part of genetics that deals with chromosomes, particularly with numerical and structural chromosome abnormalities, and their implications in congenital or acquired genetic disorders. Standard karyotyping, successfully used for the last 50 years in investigating the chromosome etiology in patients with infertility, fetal abnormalities and congenital disorders, is constrained by the limits of microscopic resolution and is not suited for the detection of subtle chromosome abnormalities. The ability to detect submicroscopic chromosomal rearrangements that lead to copy-number changes has escalated progressively in recent years with the advent of molecular cytogenetic techniques. Here, we review various gene dosage methods such as FISH, PCR-based approaches (MLPA, QF-PCR, QMPSF and real time PCR), CGH and array-CGH, that can be used for the identification and delineation of copy-number changes for diagnostic purposes. Besides comparing their relative strength and weakness, we will discuss the impact that these detection methods have on our understanding of copy number variations in the human genome and their implications in genetic counseling.

Résumé

La cytogénétique est la branche de la génétique qui a pour objet l'étude des chromosomes et vise à identifier des anomalies chromosomiques, de nombre ou de structure, impliquées dans des pathologies acquises ou congénitales. Depuis 50 ans, l'étude du caryotype standard connaît un vif succès notamment en cas d'infertilité, de handicap congénital ou de malformations fœtales. Mais l'observation microscopique des chromosomes offre une faible résolution de détection des anomalies, ce qui rend le caryotype standard inadapté à la mise en évidence de remaniements chromosomiques de petite taille. La possibilité de détecter des microremaniements à l'origine d'un déséquilibre génomique est récemment apparue avec l'avènement des techniques moléculaires de la cytogénétique. Dans cette revue sont présentées les diverses approches de quantification génique pouvant être appliquées en diagnostic pour l'identification et la caractérisation d'un déséquilibre génomique, telles que la FISH, les techniques reposant sur une PCR (MLPA, QF-PCR, QMPSF, PCR en temps réel), et la CGH sur métaphases ou sur puces à ADN. En plus de souligner leurs avantages et inconvénients respectifs, nous discutons de l'impact que ces méthodes ont sur notre compréhension des variations du nombre de copies de séquences génomiques dans le génome humain et des implications lors du conseil génétique.

Keywords: FISH; PCR-based approaches; CGH; CGH array; Copy-number variations

Mots clés : FISH ; Approches par PCR ; CGH ; CGH sur puces ; Variations du nombre de copies de séquences

* Corresponding author.
E-mail address: lgouas@chu-clermontferrand.fr (L. Gouas).

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1. Introduction

Since the first description of a chromosomal abnormality in humans by Lejeune et al. in 1959 [1], chromosome analysis remains one of the genetic tests most commonly realised for diagnostic purposes in a wide variety of indications in obstetrics and gynecology, pediatrics and oncology. Since the last 50 years, standard karyotyping has successfully identified numerical (aneuploidies) and structural (deletions, duplications, translocations) chromosome abberations in reproductive failure [2], fetal development abnormalities [3], mental retardation and/or congenital malformations [4]. Highly reliable for identifying entire chromosome aneuploidy as well as large chromosomal rearrangements, microscopic karyotype banding analysis is, however, limited to 5–10 Mb of resolution, depending on the quality of chromosome preparations.

A further and important limitation of this technique is the need for cell culture for around three days for blood samples and 1–2 weeks for fetal samples to obtain an adequate number of dividing cells for metaphase analysis. The time interval around 14 days between the collection of a prenatal sample and the reporting of karyotype results represents a time of great anxiety for parents, particularly during a high-risk pregnancy.

These two major limitations lead to the development of more rapid and resolutive techniques. Recently, molecular cytogenetic techniques have allowed the identification of gene dosage alterations involving chromosome segments smaller than 5 Mb. Fluorescence in situ hybridization (FISH) with locus-specific probes has been used to detect imbalances involved in microdeletion syndromes [5] or subtelomeric genomic rearrangements in patients with idiopathic mental retardation [6]. Furthermore, genome wide screening techniques such as comparative genomic hybridization (CGH) on metaphase chromosomes and more recently, array-based CGH, as well as several PCR-based approaches as quantitative multiplex PCR of short fragments (QMPSF), quantitative fluorescent PCR (QF-PCR), multiplex ligation probe amplification (MLPA), and real-time quantitative PCR (real-time qPCR) have been developed to detect gene dosage alterations in a variety of prenatal and postnatal disorders.

In this review, the principle of each technique will be described and their relative strength and weakness will be discussed. Then, the impact that these detection methods have on our understanding of copy number variations in the human genome and their implications in genetic counseling will be discussed.

2. Fish

FISH is a molecular cytogenetic technique in which fluorescently labeled DNA probes are hybridized to metaphase spreads or interphase nuclei. A variety of probe types is available and enables for the detection of aneuploidy and chromosome rearrangements. Particularly, locus-specific probes can be used to detect gene copy-number changes that are not generally detectable by classical cytogenetics in individuals diagnosed with a microdeletion syndrome. Indeed, FISH allowed the identification of microdeletions and microduplications in submicroscopic chromosomal regions in individuals with developmental delay and mental retardation [7]. FISH was also widely useful for acquired chromosomal abnormalities detection: gene amplification (such as *HER2* in breast cancer) or deletion (such as *p53* or *ATM* in chronic lymphocytic leukemia) are prognosis markers and response to treatment indicators [8,9].

A locus-specific probe set has been developed to simultaneously explore the entire subtelomeric chromosomal regions in humans [10–12]. These subtelomere region-specific FISH probes have further led to the identification of terminal chromosomal abnormalities in 5% of the patients with idiopathic mental retardation [6,13].

FISH is extremely useful in identifying suspected chromosome abnormalities, since these regions can be analysed with a high specificity.

However, FISH testing has several limitations. Locus-specific probes are expensive, the procedure is significantly time-consuming when using the set of subtelomere probes, and tandem microduplications may be undetected as a result of the limited resolution on metaphase spreads. Mainly, the identification of an abnormality depends on the DNA probe used, specifically on its size and hybridization localization. Indeed, small rearrangements may be missed by relatively large BAC- or PAC-probes, and some patients with atypical microdeletion/microduplication may yield normal FISH findings due to the mapping of the abnormality outside the area covered by the FISH probe. Consequence is that some patients with very small or atypical deletions/duplications will be undiagnosed by FISH testing. As example, the currently accepted clinical laboratory assay for 22q11 DiGeorge uses the TUPLE1 FISH probe, which is located within a typically deleted region of approximately 3 Mb. This assay can detect the majority of affected patients (85–90%), however, many patients with phenotypic features of 22q11 syndrome have no deletion detectable by FISH testing, and deletions that map outside the area covered by the TUPLE1 probe have been described [14,15].

Another limitation of FISH is the restrained number of chromosomal loci that can be screened in a single reaction and the requirement of high quality metaphase spreads. Thus, FISH can be efficiently used to screen for a specific rearrangement but is not suitable for high through-put diagnostic screening.

3. PCR based approaches

3.1. QF-PCR

QF-PCR methods consist of an amplification of genomic DNA sequences by PCR using fluorescent primers. The PCR products can then be visualized and quantified as peak height or area using an automated DNA sequencer and appropriate softwares. Peak heights or areas generated from the DNA of patients are generally compared to those obtained from a normal DNA. Two main QF-PCR approaches have been developed:

- the amplification of chromosome-specific, repeated DNA sequences known as short tandem repeats (STRs);
- the amplification of short exonic sequences (QMPSF).

3.1.1. QF-PCR of STRs

Short tandem repeats are stable and polymorphic, i.e., they vary in length between subjects, depending on the number of times the tri-, tetra- or penta-nucleotides are repeated. The less frequent tetranucleotide repeats have been used to investigate specific chromosomes disorders in view of their polymorphism and stability during the life of an individual [16].

On the assumption that, within the early exponential phase of PCR amplification, the amount of specific STR produced is proportional to the quantity of the initial target sequence, PCR should be performed with a limited number of cycles [17]. The optimal number of cycles must be evaluated in order to obtain, in heterozygous normal subjects, two peaks of equal fluorescent activity corresponding to the presence of two different alleles at one locus. If the STR marker is highly polymorphic, few normal subjects should be homozygous and show one peak. DNA amplified from subjects who are trisomic will exhibit either two peaks (being diallelic), one of them being twice as large or high as the other, or an extra peak (being triallelic). Samples that are diallelic, containing two peaks with area/height ratios of the shorter and the longer allele within the range 0.8–1.4, are considered to be normal. In contrast, diallelic samples with ratios < 0.65 or > 1.8, are considered to be trisomic.

Initial studies analyzed one marker at a time, but several reports described the development of multiplex assays in which 4–12 markers were co-amplified [18]. Quantitative fluorescence PCR of STRs is mainly used for rapid and simple diagnosis of aneuploidy, including trisomy 21, 13 and 18 and sex chromosome abnormalities. The first QF-PCR of STRs application involved X chromosome aneuploidy [19]. A number of reports on large series have shown the high reliability and reproducibility of the QF-PCR assay [20–22]. However, some difficulties may come from the X chromosome testing. When STRs specific for chromosome X are used, some sample from normal XX females may show homozygous patterns, indistinguishable from those produced by sample with a single X, as in Turner syndrome. Analyzing additional X-chromosome markers would reduce the likelihood of homozygosity. Furthermore, potential sources of errors must be taken into consideration when interpreting the QF-PCR results. For example, a single nucleotide polymorphism (SNP) located within the sequence covered by the primers may produce a null allele, leading to the false interpretation of a monosomy.

One advantage of QF-PCR over FISH is the possibility to detect maternal cell contamination of fetal samples. A characteristic pattern with extra alleles or skewed ratios between peaks for the target chromosomes would be seen. QF-PCR is capable of identifying autosomal mosaicism, where the trisomy is present in more than 10% of in vitro cultured cells [23,24]. Furthermore, the QF-PCR test allows the determination of the maternal or paternal origin of chromosome aneuploidies, and whether they occurred at meiosis I or II.

3.1.2. QMPSF

QMPSF is a semi-quantitative method based on a multiplex PCR of short exonic sequences. The assay consists of a simultaneous amplification by PCR, in a limited number of cycles, of short exonic fragments (< 300 pb) in a single tube, using dye-labelled primers. As for QF-PCR of STRs, the PCR is performed with a limited number of cycles so that DNA yield generated in the exponential step may be comparable between different samples, and short DNA fragments are amplified to reduce the difference in efficiency between each amplification of the assay. Moreover patented ''tags'' may be added at the 5' end of sense and antisense primers, so that it makes homogeneous the difference in melting temperatures (Tm) between the various hybridization segments used in multiplex (Patent WO/2004/009846). An additional fragment, corresponding to the exon of another gene not involved in the critical regions, is coamplified as a control. After PCR, amplicons are size differientiated by capillary electrophoresis. Electropherograms from patients are superimposed to those generated from a normal control DNA by adjusting to the same level the peaks obtained for the control amplicon. Then, the heights of the peaks corresponding to the regions to be explored are compared between the patient samples and the control DNA, and quantitative changes are simply detected by an increase or decrease of the corresponding fluorescent peaks.

QMPSF was developed in 2000 by Charbonnier et al. for the detection of deletions and duplications of mismatch repair genes in hereditary nonpolyposis colorectal cancer syndrome, and has been used to screen for genomic dosage variations in genes like CFTR [25]. More recently, comparison of a QMPSF method with FISH for the assessment of the four aneuploidies commonly evaluated in chronic lymphocytic leukemia patients (namely deletions of the 11q22, 13q14, 17q13 regions and trisomy 12) demonstrated that QMPSF was a cost-effective method perfectly adapted to the detection of primary defect [26]. Saugier-Veber et al. validated the application of QMPSF to explore simultaneously 12 candidate loci known to be the target of genomic rearrangements and involved in mental retardation [27]. Thus, QMPSF has been shown to be a sensitive method for the detection of both deletions and duplications and was recently used to validate copy-number change variations (CNVs) detected in the human genome by array CGH [28].

QMPSF is a rapid and relatively cheap method to detect genomic rearrangements. Furthermore, QMPSF is a one step assay, i.e., PCR amplification before capillary electrophoresis. This feature has the advantage to minimize the risk of sample crosscontamination and should facilitate full automation of the assay. Furthermore, the flexibility of the QMPSF method will allow the gradual integration of new candidate loci recognised as deleted or duplicated in pathologies. This flexibility also considerably facilitates, in each patient harbouring a rearrangement, the delineation of the boundaries.

Conversely, a single QMPSF reaction contains no more than 15–20 targets, and it is unclear whether this number might be increased without affecting the sensitivity of the assay. As for QF-PCR, potential misinterpretation of QMPSF may result

from the presence of a SNP or a single point mutation in sequences covered by the primers.

Because this method is based on the comparison of electrophoregrams and is not quantitative, the critical point is the quality of the DNA, which is demonstrated by the fact that sometimes electrophoregrams generated from DNA extracted according different methods could not be superimposed.

3.2. MLPA

MLPA was first described in 2002 by Shouten et al. and was designed to detect gene dosage abnormalities in a wide range of conditions by the relative quantification of up to 45 different DNA sequences in one reaction [29].

In contrast to QF-PCR of STRs and QMPSF, it is not genomic sequences but the probes added to the samples that are amplified and quantified. Each MLPA probe consists of two oligonucleotide hemiprobes, one synthetic and one derived from the single-stranded bacteriophage M13. These oligonucleotides hybridize to adjacent sites of the target sequence. Each hemiprobe has a universal primer sequence on one end and one has a stuffer sequence that leads to PCR products with different and unique length. Once hybridized, the two hemiprobes are joined by a ligase, and the probe can then be amplified by PCR. The ligation products are PCR amplified using a single universal dye-labelled primer pair and PCR products of unique size (130–480 bp) can be then separated by capillary electrophoresis. The relative quantity of each of the PCR products is proportional to the number of copies of target sequence. Results are given as allele copy numbers as compared to normal controls: a ratio of about 1 is obtained if both alleles are present, a ratio of about 0.5 if one allele is absent and a ratio of about 1.5 if one allele is duplicated.

Many studies have shown that subtelomeric aberrations are a significant cause of idiopathic moderate to severe mental retardation, and subtelomeric FISH probes are the most commonly used technique in detecting such abnormalities [12]. However, the use of a complete set of subtelomeric FISH probes is laborious, time-consuming and costly. Several laboratories have therefore developed the MLPA strategy to explore subtelomeric regions in patients with mental retardation [30]. Recent studies have also assessed the use of MLPA with subtelomeric probe mixtures in spontaneous miscarriages and have confirmed the use of this approach to detect aneuploidy and unbalanced terminal chromosomal rearrangements in miscarriages [31,32]. Prenatal detection of aneuploidy involving chromosome 13, 18, 21, X and Y by MLPA with the appropriate probe mixture has also been reported. It was concluded that aneuploidy screening in uncultured amniocytes by MLPA was feasible in a clinical diagnostic setting [33–35].

Many other commercial kits for MLPA assays are available from MRC Holland and have a variety of applications including detection of mutations [36] and single nucleotide polymorphisms [37], analysis of DNA methylation [38], relative mRNA quantification [39], chromosomal characterisation of cell lines and tissue samples [40], detection of duplications and deletions in human cancer predisposition genes such as *BRCA1*, *BRCA2*, *hMLH1* and *hMSH2* [41]. A comparison of MLPA and FISH combined with automated spot counting in detection of *HER2* amplification in breast carcinomas revealed that the overall concordance of detection of *HER2* gene amplification was 98%: MLPA is then a reliable and reproducible technique and can be used as an either alternative or additional test to determine *HER2* status in breast carcinomas [42].

MLPA has rapidly gained acceptance in genetic diagnostic laboratories due to its simplicity and high sensitivity compared to other methods, relatively low cost, capacity for reasonably high throughput and its robustness. For example, MLPA allows the relative quantification of up to 46 different DNA targets in a single reaction with results typically available after 2–3 days, and is particularly less labour-intensive than subtelomeric FISH.

However, MLPA encompasses some limitations. One of these is its inability to detect maternal cell contamination and ploidy changes. Another one is that MLPA reactions are more sensitive to contamination and PCR inhibitors such as small remnants of phenol and could explain the failure of some assays. Furthermore, point mutations or SNP which lie close to the ligation site of a MLPA probe may affect the efficient ligation of the probe, leading to an apparent reduced relative peak height and a potential misinterpretation.

The presence of CNVs throughout the genome has been widely demonstrated [28,43,44]. When analysing subtelomeric regions with two different dedicated MLPA probe mixtures, positive results, obtained with only one of the two probes, are more likely to be considered as CNVs. Thus, the existence of subtelomeric copy number polymorphisms renders testing samples with two probe mixtures indispensable (P036D, P070). Nevertheless, in addition to comparing the results with those of the parents, techniques such as FISH, QF-PCR, QMPSF, or quantitative real-time PCR should be employed in order to confirm MLPA results.

3.3. Real-time qPCR

Real-time qPCR-genotyping relies on the fact that the fractional cycle number (Ct), at which the amount of an amplified target (amplicon) reaches a fixed threshold, is directly related to the amount of starting target [45,46]. A higher or lower starting copy number of the genomic DNA target will result in a significant earlier or later increase in fluorescence, respectively, and thus, in a decreased or increased Ct. The accumulation of PCR product can be monitored by staining using different detection systems, either based on hybridization probes such as TaqMan [46–48], Scorpions [49], FRET probes [50], or on intercalation by fluorescent dyes, such as the ds-DNA binding dye SYBR Green I (Roche Diagnostics) [51–53]. Real-time qPCR experiments are performed in a thermal cycler that incorporates an optical system for excitation of fluorochromes and monitoring of emitted wavelengths of light.

Use of intercalating dyes, while economical, poses the risk that fluorescence signals could be due to non-specific double-stranded product. Fluorescent probes reduce that risk and offer the prospect of multiplexing through the use of more than one

fluorochrome. Nevertheless, given that the synthesis of fluorescence-labelled probes is still a major cost factor, the use of a general detection method is of great benefit. The SYBR Green I-based quantification is a method of choice to combine the sensitivity, accuracy, and reproducibility of real-time qPCR, with the low cost, simplicity, and flexibility of the SYBR Green I detection [54].

Some investigations using real-time qPCR for the detection of chromosomal deletions and duplications have been reported. Weksberg et al. presented a real-time qPCR approach for the detection of chromosomal microdeletions and microduplications at cytoband 22q11 in patients with Velocardiofacial Syndrome [55], and some reports have shown the sensitivity of real-time qPCR in detecting subtelomere chromosomal rearrangements [56,57]. Real-time qPCR was also proven to be a valuable method for the analysis of single copy genes [58–60]. Furthermore, real-time qPCR may also be helpful in malignancies to identify amplification, such as *IL-6* gene which is amplified and overexpressed in human glioblastomas [61].

Real-time qPCR offers a number of potential advantages over other systems. Real-time qPCR provides a mean for continuous detection of products throughout the amplification process, and as such can dispense with a gel separation stage, thus saving substantial technical time, and operate in a closed system, eliminating the danger of PCR contamination and sample crosscontamination. The use of SYBR Green I dye for the detection of PCR-products allows the use of conventional and low-cost oligonucleotides for the amplicon design and the primer-set can easily be extended without any technical restriction to perform gene dosage measurement of a specific chromosomal region or detailed molecular characterization of deletion/translocation breakpoints. However, the existence of repetitive and genome-wide amplified elements may be problematic in narrowing down the resolution of deletions/translocation breakpoint-mapping.

However, real-time qPCR presents disadvantages too. Using hybridization probes, the number of loci that can be analysed in a single tube is currently restricted by fluorescence profiles and by optical properties of hardware to four or less. Thus, in applications where multiple loci have to be examined, this could be done only in multiple reactions. The cost of probes could also become prohibitive in some applications. While highly sensitive to demonstrate 3:2 dosage differences, the real-time qPCR sensitivity is limited in mosaic cases, where it will probably failed to detect low-grade mosaicism.

4. CGH

CGH is a molecular cytogenetic technique in which differentially fluorescently labelled patient and reference whole-genomic DNA are hybridized on control metaphase chromosome slides. For each chromosome, a ratio value of the fluorescence intensity is generated from five to 20 metaphase spreads. Thus, differential hybridization signals allow the detection of unbalanced gains and losses of chromosomal material across the whole genome [62–64]. CGH has been useful for the analysis of chromosomal imbalances in solid tumours. Recurrent patterns of gains and losses have been observed in the malignant tissue from numerous human cancers [65–68]. Although the size resolution of CGH is lowest near the telomeres, the technique has allowed detecting unbalanced translocations not detectable on standard karyotype in patients with idiopathic mental retardation and dysmorphic feature [69,70]. Thus, CGH may be realised in routine for patients with a variety of conditions for whom no chromosomal aberrations have been previously detected by karyotype and/or specific FISH probes. Nevertheless, CGH is limited by a resolution of 3–10 Mb and aberrations with smaller size will not be detected.

5. Array-based CGH

A new molecular cytogenetic technique, array CGH analysis, has recently been developed. Although based on the same principle as conventional CGH, array CGH differs in that genomic clones from selected regions of the genome replace the normal control metaphases as the target DNA [71,72]. Since genomic clones are used as the target DNA, the resolution of the technique is theoretically increased and depends on the size and the density of target DNA. Therefore, rearrangements that are not visible by conventional CGH can be detected.

Targets for array CGH can be large insert clones (cosmid, P1, PAC, BAC) [71] or oligonucleotides [73]. Lower-density array with BAC clones were first used since targeted clones were currently implicated in cytogenetics diagnosis. This was the first generation array CGH which only contains 287 clones unevenly distributed on the genome. This array was able to screen all human subtelomere regions, microdeletion syndromes, oncogenes and tumor suppressor genes [74]. Array CGH covering a whole chromosome [75], a chromosome segment [76], all subtelomeric regions [77], or the entire genome at a 1 Mb [78] and at a 10–100 Kb resolution level [79] were also developed. Array with overlapping clones, named tiling path arrays, have also been constructed.

Array CGH has been successfully utilized to identify amplifications and deletions in numerous malignancies [80] and in constitutional disorders [77,81–83].

One of the first applications of array CGH was to screen for subtelomeric rearrangements in patients with developmental disorders [77,84]. Then, several studies validated array CGH to detect cryptic chromosome imbalances by using positive controls with well-known and characterized anomalies [85–87]. Array CGH is also useful to map and delineate precisely the size and breakpoints of chromosomal aberrations. Using this approach, cryptic deletions or duplications were identified in patients with de novo apparently balanced chromosome rearrangements. Array CGH results also permit to describe a 5 Mb critical region on chromosome 18q22.3-q23 in congenital aural atresia [88]. Furthermore, by identifying a recurrent deleted region in patients with CHARGE syndrome, array CGH has made possible to identify the *CHD7* gene as causal after loss of function mutations were described in this gene [89].

Development of array CGH in routine for screening in haematological disorders is increasing: for example, a targeted DNA microarray for clinical evaluation of recurrent abnormalities in chronic lymphocytic leukemia (CLL) has been recently validated. The author's results suggest that a subset of potentially significant genomic alterations in this pathology is being missed by FISH. Furthermore, this pilot study clearly showed the robustness, high sensitivity, and high specificity for the targeted CLL microarray analysis [90].

The main advantage of array CGH is the ability to explore hundreds or thousands of discrete loci in a single assay and to detect any DNA dosage imbalance including aneuploidies, deletions and duplications with a resolution much higher than karyotype and conventional CGH.

However, array CGH could not characterize the structural configuration of the abnormal chromosomes, particularly, the order and orientation of the rearranged segments. Furthermore, array CGH is unable to detect polyploidies, and low-level mosaicism may be difficult to detect. But the major limitation of array CGH is interpretation difficulties. Most of the false negative results may be due to technical problems resulting from poor hybridization or imperfect coverage of the genomic region of interest [91]. Due to the risk of false positive results, CNVs detected by array CGH should be validated with another technique such as FISH, QMPSF or real-time qPCR. A replicate dye-swap experiment where fluorochromes are inverted between patient and control has been proposed to decrease the number of false positive results [91,92]. Interpretation difficulties result also from the presence of CNVs with unclear clinical significance in the human genome. Depending on the resolution and representation of the genome of the microarray, CNVs may be frequently found. For example, in a report of 100 children with mental retardation using a high-density oligonucleotide array, 11 were found to have de novo copy-number changes that were considered to be clinically relevant. But, on average, 30 CNVs were detected in every child of the study [93]. A better knowledge of these polymorphisms would be required before applying array CGH in a diagnostic setting. In this view, several databases have been initiated, like the DECIPHER database (http://www.sanger.ac.uk/PostGenomics/decipher) and database of genomic variants (http://projects.tcga.ca/variation/), in order to list and map genomic polymorphic segments.

6. Discussion

The considerable gap in resolution between traditional cytogenetic techniques (megabase pairs) and molecular biology techniques (base pairs) has been closed to a large extent by molecular cytogenetic techniques. Indeed, a wide range of methods are now available for gene dosage measurement in cytogenetic purposes, such as FISH, QF-PCR, QMPSF, MLPA, CGH and more recently array CGH. Except for FISH, these methods were proven to be more rapid, resolutive and less labour-intensive than conventional karyotyping.

While FISH, QF-PCR, QMPSF, MLPA and CGH have been validated with large series of patients and are currently implemented in diagnostic laboratories due to their low-cost and simplicity, high-resolution array CGH need further evaluation before its transfer in a routine clinical setting. In particular, the major difficulty will be in prenatal diagnosis because in the absence of a perfect knowledge of the phenotype to which the copy number alteration could be correlated, the array results would be less informative and would provoke anxiety to the parents.

The development of these molecular cytogenetic technologies has increased the ways to detect chromosomal abnormalities in pre- and postnatal disorders. These technologies have improved the detection rate of genomic imbalances as a causative factor in different disorders, such as in moderate to severe mental retardation with or without dysmorphology [70,94], and the description of new chromosomal disorders.

However, the high-resolution of some of these techniques raises the issues of interpretation difficulties, particularly when discriminating between pathologic and benign copy-number alterations. Clinical significance of an aberration may depend on different criteria:

- the previous identification of the abnormality in an individual with the same phenotype;
- the de novo origin;
- the size of the aberration, larger imbalances are more likely to be pathogenic.

However some examples showed the complexity of the relationship between chromosomal imbalances and the phenotype. Cytogenetically visible imbalances (> 5 Mb) were detected in phenotypically normal parents of probands [95,96] and de novo detected aberrations may represent de novo occurrence of a CNV, even if the frequency of de novo CNVs is unknown. Furthermore, one has to consider that a chromosomal abnormality may also be of clinical significance even though it has been inherited from a healthy parent because more complex mechanisms such as phenotypic variation, incomplete penetrance, effect of imprinted genes, position effect or a point mutation in a recessive gene may be involved.

Our understanding of benign and pathogenic genomic variation is still in its infancy, and publicly available databases, such as DECIPHER and database of genomic variants will be helpful in assessing the potential involvement of CNVs in identified alterations. Moreover, a careful phenotypic assessment and literature search would help to evaluate the clinically relevance of chromosomal abnormalities detected by these techniques. Further research into the discovery and characterization of human CNVs is needed to develop more comprehensive human genetic variation maps and to facilitate more accurate interpretations of the clinical impact of genomic imbalances. This will help clinicians in the genetic counseling and to implement the use of high-resolution, genome-wide array CGH assays not only for diagnosing genetic disorders in individuals with dysmorphism and developmental delay but also for prenatal genetic diagnosis.

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2ème partie

2ème PARTIE : Corrélations génotype-phénotype à partir de plusieurs cas cliniques

I/ Introduction - Revue de la littérature

Il existe une variabilité du phénotype pour toutes les anomalies chromosomiques. En effet, en cas d'anomalies chromosomique déséquilibrée, si l'effet de dosage génique est responsable en grande partie des altérations du phénotype (Epstein *et al*, 1990 ; Korenberg *et al*, 1990), d'autres mécanismes, aboutissant à une régulation complexe de l'expression des gènes, peuvent être également impliqués.

La trisomie 21 ou syndrome de Down, est la plus fréquente des anomalies chromosomiques constitutionnelles viables (1/700), et donc la plus étudiée. La variabilité phénotypique du syndrome de Down concerne de nombreux signes cliniques de la maladie : l'importance du retard intellectuel, la présence inconstante d'une cardiopathie ou d'un pli palmaire transverse unique mais également, très précocement dans le développement, la présence fréquente mais pas constante d'une anomalie de l'épaisseur de la clarté nucale, très bon signe de dépistage. Pour expliquer cette variabilité et le fait que certains des traits phénotypiques de la trisomie 21 existent dans la population générale (le pli palmaire transverse unique par exemple) ou dans d'autres trisomies, l'hypothèse d'une dérégulation secondaire de nombreux autres gènes du développement situés sur d'autres chromosomes a été proposée (Shapiro, 1983 ; Shapiro, 1997 ; Pritchard et Kola, 1999). Des études réalisées chez un modèle de souris porteuse du syndrome de Down (Ts65Dn) ont montré que seulement un tiers à la moitié des gènes du chromosome 21 avaient une augmentation du niveau de leurs transcrits, les autres échappant à l'effet de dosage génique (Kahlem *et al*, 2004 ; Lyle *et al*, 2004). Récemment, Prandini *et al* (2007) ont comparé l'expression d'environ 130 gènes du chromosome 21 sur des lignées cellulaires lymphoblastoïdes et de fibroblastes de patients avec un syndrome de Down et de patients sains. Seulement 39% et 62% des gènes, sur les lignées lymphoblastoïdes et sur les fibroblastes respectivement, étaient exprimés différentiellement. Trois groupes de gènes ont été définis : 1/ ceux toujours surexprimés, probablement impliqués dans les traits phénotypiques constants du syndrome de Down comme le retard mental, l'hypotonie ou l'Alzheimer précoce ; 2/ ceux avec un niveau d'expression variable qui pourraient être responsables de la variabilité phénotypique des patients trisomiques 21 ; 3/ ceux dont l'expression est identique chez les patients trisomiques 21 et les patients sains, donc insensibles à l'effet de dosage génique. Cependant certains gènes du dernier groupe, comme le gène *APP* (amyloid precursor protein) connu pour être responsable de la maladie

d'Alzheimer, peuvent avoir une expression tissus-spécifique (le cerveau pour APP) d'où l'intérêt de réaliser ce type d'études dans divers tissus. L'étude de Altug-Teber *et al* (2007) réalisée à partir de cellules trophoblastiques et d'amniocytes en culture montre que seulement 9% (cellules trophoblastiques) et 13% (amniocytes) des gènes du chromosome 21 sont surexprimés. De plus, les gènes surexprimés ne sont pas les mêmes dans les deux types cellulaires. Mao *et al* (2005) ont étudié l'expression de 15 000 gènes dans divers types cellulaires issus de tissu cérébral et cardiaque et ont également montré que la surexpression de certains gènes était tissu spécifique. L'ensemble de ces résultats laisse supposer qu'il existe une régulation complexe, tissus spécifique, de l'expression des gènes et pouvant être expliquée par l'existence de facteurs de compensation de dosage comme des ARN non codants (Dermitzakis *et al*, 2002 ; Antonarakis *et al*, 2004). La complexité de ces mécanismes moléculaires est bien démontrée dans les travaux de Rachidi et Lopes (2007 ; 2008) proposant un nouveau modèle moléculaire et cellulaire pour expliquer la variabilité du retard mental dans le syndrome de Down.

L'hypothèse d'une dérégulation pangénomique secondaire a également été étudiée dans les trisomies 21, 13 et 18 à un stade précoce du développement fœtal (Altug-Teber *et al*, 2007). Comme pour la trisomie 21, seulement 12 % des gènes des chromosomes 13 et 18 sont surexprimés en cas de trisomie par rapport aux fœtus témoins. Contrairement aux trisomies 13 et 21 qui n'entrainent que peu de modifications transcriptionnelles des gènes portés par les autres chromosomes, la trismie 18 serait à l'origine d'une dérégulation transcriptionnelle pangénomique importante.

Le syndrome de Di George (MIM 188 400 et 192 430) ou microdélétion 22q11 (1/4 000 naissances) est un autre exemple de syndrome ayant une grande variabilité phénotypique inter- et même intrafamiliale malgré la taille quasi-constante de la microdélétion responsable du syndrome (Lindsay *et al*, 1995 ; Ryan *et al*, 1997 ; Scambler, 2000). Cette variabilité a même été retrouvée à plusieurs reprises chez des jumeaux monozygotes (Goodship *et al*, 1995 ; Fryer, 1996 ; Hatchwell, 1996 ; Yamagishi *et al*, 1998 ; Vincent *et al*, 1999 ; Lu *et al*, 2001). En 2001, Lyndsay *et al* ont montré que le gène TBX1 (codant pour un facteur de transcription à boite T), fortement exprimé à proximité des artères des arcs pharyngés, pouvait, en cas d'haploinsuffisance rendre compte des cardiopathies conotroncales observées chez les patients (Lindsay *et al*, 2001). Pour expliquer la présence inconstante de la cardiopathie, Stalmans *et al* (2003) ont montré chez la souris, que *VEGF (*vascular endothelial growth factor), régulateur majeur de l'angiogenèse, pourrait intervenir comme gène modificateur et expliquer la présence ou non de cardiopathie en fonction de la présence de certaines isoformes. Chez la souris, il

existe au moins trois isoformes: $Vegf^{120}$, $Vegf^{164}$ et $Vegf^{188}$. L'expression de TBX1 est réduite en l'absence de l'isoforme $Vegf^{164}$ et les souris $Vegf^{164-}$ présentent des anomalies tout à fait comparables aux symptômes du syndrome de Di George. Chez zebrafish, une diminution de la quantité de VEGF aggrave les malformations artérielles induites par l'invalidation de *TBX1*. Il semble donc que l'explication de la grande variabilité phénotypique du syndrome de Di George se trouve en 6p12, localisation chromosomique de VEGF.

Ces études nous montrent la complexité des mécanismes génétiques pouvant être à l'origine d'un phénotype et de sa variabilité. A cela viennent s'ajouter d'autres facteurs épigénétiques et environnementaux.

Enfin, certaines anomalies chromosomiques apparemment équilibrées peuvent être associées à une altération du phénotype. Des microdélétions ou microduplications au niveau des points de cassure peuvent être à l'origine du phénotype anormal. Ces déséquilibres peuvent être mis en évidence par CGH array. Les autres mécanismes pouvant expliquer l'altération du phénotype associée à ces remaniements équilibrés sont la présence d'un point de cassure dans un gène fonctionnel ou d'un effet de position Cependant il est difficile de prouver leur implication et le conseil génétique, notamment quand l'anomalie survient « de novo » et est de découverte prénatale, est alors très délicat.

Dans ce travail, nous proposons de discuter à travers plusieurs cas publiés, les hypothèses pouvant expliquer de telles discordances génotype-phénotype.

II/ Anomalie chromosomique déséquilibrée à phénotype variable

1) A propos d'un isochromosome 20q en mosaïque (Goumy *et al*, 2005, Publication n°4)

La présence en mosaïque d'un isochromosome 20q [i(20q)] est classiquement considérée comme sans conséquence phénotypique (Chen CP, 2003). Les différentes observations prénatales amènent à la conclusion que l'anomalie chromosomique serait le plus souvent confinée aux tissus extra-embryonnaires ce qui expliquerait l'issue favorable de la grande majorité des grossesses. Seulement trois cas d'i(20q) en mosaïque associé à un phénotype anormal ont été publiés (Chernos *et al*, 1992 ; Pfeiffer *et al*, 1997 ; Chen CP, 2003) et les auteurs concluaient que les malformations observées, différentes dans les 3 cas, n'étaient vraisemblablement pas en relation avec l'anomalie chromosomique.

Nous décrivons un cas d'i(20q) en mosaïque de découverte anténatale associé à un syndrome polymalformatif comprenant une hydrocéphalie, des pieds bots et des malformations vertébrales. Une interruption thérapeutique de grossesse a été proposée. L'examen fœtopathologique retrouvait, en plus des malformations vues à l'échographie, une dysmorphie faciale, une hypoplasie cérébelleuse majeure et des anomalies oculaires. Les radiographies du fœtus montraient une anomalie de segmentation des vertèbres thoraciques. Le caryotype standard en bandes G et en bandes R, réalisé à partir d'une culture d'amniocytes, a mis en évidence la présence d'un i(20q) dans 14 clones sur 15. Par hybridation in situ en fluorescence (FISH) avec une sonde locus spécifique (LSI 20q12) en direct, l'anomalie n'était retrouvée dans aucun des 20 noyaux interphasiques analysés : la culture semble donc avoir favorisé la prolifération des cellules anormales. Le caryotype des fibroblastes n'a pas pu être réalisé en raison d'une absence de prolifération des cellules en culture. Une CGH sur métaphases et une CGH sur puce (ABBOTT, 287 clones) ont été réalisées afin d'éliminer un autre déséquilibre génomique pouvant être à l'origine des signes cliniques. Ces deux analyses n'ont pas mis en évidence d'anomalie supplémentaire.

Certaines malformations retrouvées chez ce fœtus, en particulier les anomalies oculaires, les pieds bots et les anomalies de segmentation vertébrale, sont comparables à celles décrites par Pfeiffer *et al* (1997). Des malformations vertébrales sont également retrouvées dans les délétions du bras court du chromosome 20. Tous ces éléments sont en faveur d'une relation directe entre les malformations retrouvées chez le fœtus et la présence de l'isochromosome 20q à l'origine d'une trisomie 20q et d'une monosomie 20p.

Dans l'article de Pfeiffer *et al* (1997), l'i(20q) était retrouvé dans 24 clones sur 26 au caryotype et dans seulement 6% des noyaux interphasiques sur frottis buccal. Le phénotype correspondant à cette anomalie en mosaïque ne semble donc pas lié au pourcentage de cellules anormales qui avant culture, dans notre cas comme dans celui décrit par Pfeiffer, est très faible.

La découverte en prénatal d'un isochromosome 20q en mosaïque doit donc inciter à la prudence lors du conseil génétique et faire rechercher des malformations associées à l'échographie.

2) A propos d'un syndrome de Larsen atypique (Goumy *et al*, 2008, Publication n°5)

Le syndrome de Larsen (LS) est caractérisé par des luxations multiples et une dysmorphie faciale. Il existe une grande variabilité clinique et des cas familiaux ont été

décrits avec une transmission autosomique dominante (OMIM 150250) ou récessive (OMIM 245600). Récemment, des mutations dans le gène *FLNB* (filamine B) localisé en 3p14.3 ont été rapportées dans des cas de LS (Zhang *et al*, 2006 ; Bicknell *et al*, 2007).

Nous rapportons le cas d'un fœtus présentant les caractéristiques cliniques et radiologiques du LS et porteur d'une translocation déséquilibrée 3;5 à l'origine d'une trisomie partielle 3p et d'une monosomie 5p. Cette anomalie chromosomique, invisible au caryotype standard malgré la taille importante des segments remaniés, a été mise en évidence par CGH sur métaphases. Le gène *FLNB* étant localisé à proximité du point de cassure sur le chromosome 3, nous avons émis l'hypothèse que la présence en 3 copies de *FLNB* pouvait être à l'origine du phénotype « Larsen-like ». La FISH à façon, réalisée à l'aide d'une sonde fabriquée à partir d'un BAC ciblant la région de *FLNB*, a montré la présence de *FLNB* en seulement deux exemplaires et nous a permis d'exclure l'hypothèse d'un effet de dosage génique pour expliquer les anomalies squelettiques du fœtus. Cependant, des mécanismes de dérégulation plus complexes comme la surexpression d'enhancers situés en amont de *FNLB*, peuvent être à l'origine du phénotype. Nous envisageons donc d'étudier l'expression du gène *FLNB* chez ce fœtus à partir des prélèvements cryoconservés.

Seulement trois cas de LS associés à des anomalies chromosomiques déséquilibrées ont été décrits (Pierquin *et al*, 1991 ; James *et al*, 2003). Une monosomie 6p distale était présente dans les 3 cas suggérant la présence d'un locus « Larsen-like » en 6pter. A notre connaissance il s'agit du premier cas de LS associé à un déséquilibre impliquant les bras courts des chromosomes 3 et 5.

3) A propos d'un chromosome 9 atypique (Goumy *et al*, 2005, Publication n°6)

Les variants du chromosome 9, liés au polymorphisme de la taille de la constriction secondaire et à l'existence d'inversions péricentriques limitées à cette constriction secondaire, sont fréquents et considérés comme non-pathologiques.

Nous rapportons le cas d'un enfant de 10 ans présentant un retard de langage, une légère dysmorphie faciale et un retard statural (-2DS) dont le caryotype a été établi à 46,XY,der(9) avec un chromosome 9 remanié au niveau de la région centromérique et de la constriction secondaire mais n'ayant pas l'aspect d'un 9qh+ ou d'un inv(9)(p11q12) classiques.

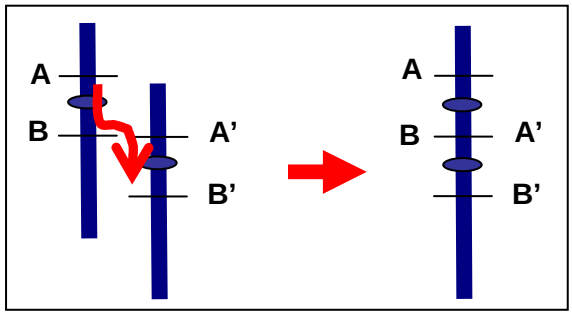
Par FISH, nous avons montré que les deux chromosomes 9 étaient entièrement peints par la sonde WCP 9 et que le chromosomes der(9) était dicentrique (sonde CEP 9).

La CGH a mis en évidence une amplification des régions p(11)p(12) et q(12)q(21). Ces différents résultats ont conduit à proposer la formule chromosomique suivante : 46,XY,der(9)(pter→q21::p12→q21::q21→qter). Le dérivé 9 correspond donc à un chromosome 9 dicentrique dont la région p12→cen→q21 est dupliquée.

Une première enquête familiale a permis de mettre en évidence la présence d'un chromosome 9 remanié comparable chez le père à phénotype « normal » et chez le frère de Mathieu présentant un léger retard des acquisitions, avec notamment une dysphasie.

Un mésappariement méiotique à l'origine d'un crossing-over inégal durant la première division méiotique est probablement à l'origine de cette anomalie puisqu'il existe des séquences homologues au niveau des bandes p12 et q13q21.1 (Starke *et al*, 2002). Ce mécanisme a été proposé par Lukusa *et al* (2000) rapportant un chromosome 9 « variant » semblable à celui décrit ici et observé chez une patiente de 31 ans présentant un retard mental et une dysmorphie faciale comparable à celle de notre patient avec notamment une grande bouche, un philtrum court (Fig. 4). Une variabilité phénotypique intrafamiliale était également rapportée dans le cas de Lukusa puisque la sœur de la patiente était porteuse « asymptomatique » du même chromosome 9.

FIGURE 4 : Mésappariement méiotique conduisant au chromosome 9 dicentrique d'après Lukusa et al. 2000



Puisque aucun autre déséquilibre n'a été mis en évidence par CGH et qu'un cas similaire a déjà été décrit, nous pensons que la duplication 9p observée est à l'origine du phénotype du patient. La variabilité phénotypique observée dans cette famille peut être expliquée par un effet de position. En effet, le segment dupliqué est encadré par l'hétérochromatine de la constriction secondaire et les régions codantes situées entre les deux centromères pourraient donc être inactivées de façon variable.

En conclusion, lorsque le caryotype standard met en évidence un chromosome 9 remanié au niveau de la région péricentromérique et s'il existe des manifestations

phénotypiques associées, des investigations cytogénétiques plus poussées doivent être envisagées.

III/ Anomalie chromosomique équilibrée à phénotype altéré

A propos d'un remaniement chromosomique complexe (Goumy *et al*, 2006, Publication n°7)

Les anomalies de structure équilibrées sont relativement fréquentes dans la population. Les plus communes sont les translocations robertsonniennes et réciproques (incidence d'environ 1/1 000 pour chacune). Ces anomalies sont souvent à l'origine de troubles de la reproduction et sont donc dépistées essentiellement lors de bilan d'hypofertilité ou de fausses couches à répétition chez des couples sans altération du phénotype (Portnoï *et al*, 1988). En prénatal, dans la plus grande étude réalisée, l'incidence des translocations réciproques de novo « apparemment » équilibrée était de 1/2 000 amniocentèses, celle des translocations robertsonniennes de 1/9 000 et celle des inversions de 1/10 000 avec un risque cumulé de phénotype anormal ou de retard du développement de 6,7% (Warburton, 1991). Une étude plus récente faite sur plus de 12 000 amniocentèses retrouvait une incidence de 2,5‰, pour les translocations réciproques (0,8‰ *de novo*), 0,8‰ pour les robertsonniennes (0,16‰ *de novo*) et 1,9‰ pour les inversions (0,24‰ *de novo*) (Peng *et al*, 2006). Dans cette étude, des signes d'appels échographiques et/ou un phénotype anormal étaient retrouvés avec un taux de 1,96% pour les anomalies héritées et de 6,66% pour les anomalies *de novo*, ce qui est supérieur au taux retrouvé dans la population générale (1,4%). Ceci montre l'intérêt d'un examen échographique détaillé et d'un suivi échographique rapproché en cas de mise en évidence de façon fortuite d'un remaniement équilibré en prénatal et surtout de la nécessité de réaliser le caryotype des parents, les remaniements hérités étant de meilleur pronostic.

Ces anomalies sont dites « apparemment » équilibrées au caryotype standard car elles ne présentent ni perte ni gain en matériel chromosomiques visibles. Avec le développement des techniques moléculaires plus résolutives, notamment la FISH à façon à l'aide de BAC ou de YAC ou la CGH array, des microdéséquilibres ont pu être mis en évidence au niveau des points de cassures de ces remaniements *de novo* « apparemment » équilibrés chez des patients à phénotype altérés (Wirth *et al*, 1999 ; Li *et al*, 2008). Ainsi, Astbury *et al* (2004) ont étudiés 15 patients porteurs de réarrangements « apparemment » équilibrés associés à un phénotype anormal et mis en évidence par caryotype haute résolution et FISH ciblant les points de cassure des délétions de 0,8 à 15,3 Mb chez 9 d'entre eux. Pour 5 des 6 autres cas, les auteurs pensent que le point de cassure se trouve

au niveau d'un gène important dont le défaut d'expression serait responsable du phénotype. Dans l'étude de Gribble *et al* (2005), un déséquilibre a été retrouvé chez 6 patients sur 10 grâce à une puce de résolution de 1 Mb : 3 d'entre eux étaient situés au niveau ou à proximité des points de cassures et les 3 autres sur d'autres chromosomes non impliqués dans le remaniement. Ciccone *et al* (2005) ont étudié par CGH array quatre patients présentant un retard mental et diverses anomalies phénotypiques porteurs de translocations « apparemment » équilibrées. Dans tous ces cas il s'agissait en fait de remaniements complexes et pour 3 d'entre eux un déséquilibre a été mis en évidence, au niveau du point de cassure dans un cas et ailleurs dans le génome pour les 2 autres cas. Une autre étude a montré qu'un pourcentage élevé (18%) de translocations réciproques apparemment équilibrées associées à un phénotype anormal étaient en fait des remaniements complexe, c'est-à-dire avec au moins 3 points de cassure (De Gregori *et al*, 2007). Sismani *et al* (2008) ont étudié 12 patients à phénotype altéré et porteurs de translocations apparemment équilibrées, 6 étant héritées et 6 *de novo*. Dans 3 cas sur 12 (2 *de novo* et 1 hérité), des microdéséquilibres allant de 2.3 à 10 Mb ont été mis en évidence au niveau ou près des points de cassure grâce à une puce de résolution de 1 Mb. Baptista *et al* (2008) ont voulu rechercher de tels déséquilibres cryptiques chez des patient porteurs d'un remaniement équilibré avec (14) ou sans (31) altération du phénotype. Les 4 déséquilibres mis en évidence dans cette étude concernaient les patients à phénotype altéré. Enfin, l'étude la plus récente portant sur 42 patients porteurs d'une anomalie chromosomique équilibrée associée à un retard mental mettait en évidence par CGH array 40% de délétions cryptiques allant de 60 kb à 15 Mb (Schluth-Bolard *et al*, 2009). La proportion de ces délétions était la même qu'il s'agisse d'un remaniement hérité ou *de novo*.

Nous décrivons ici un cas de translocation réciproque « apparemment » équilibrée au caryotype standard mais qui s'est révélée complexe lors de la caractérisation moléculaire plus poussée, réalisée en raison du retard des acquisitions et des troubles psychotiques du patient. Ce cas souligne l'intérêt de confirmer tout remaniement « apparemment » simple, réciproque et équilibré par des techniques moléculaires de cytogénétique. Ces remaniements complexes sont définis par la présence d'au moins 3 points de cassure et échange de matériel entre deux chromosomes ou plus (Pai *et al*, 1980). Ils sont rares en pathologie constitutionnelle et souvent responsable d'une altération du phénotype par pathologie du point de cassure. En effet, le risque de malformation / retard mental serait directement lié au nombre de points de cassure (Ruiz *et al*, 1996 ; Lespinasse *et al*, 2004). Dans le cas que nous avons décrit, aucun déséquilibre n'a été mis en évidence

par CGH et CGH sur puce (ABBOTT, 287 clones), ni au niveau des 4 points de cassures, ni ailleurs dans le génome mais la résolution de la CGH ne permet pas de mettre en évidence des microremaniements et la puce utilisée ne couvrait pas tout le génome. Dans ce cas, nous supposons que le retard psychomoteur du patient est dû soit à un microdéséquilibre au niveau d'un des points de cassure soit à la dérégulation fonctionnelle d'un gène qui peut être coupé au niveau d'un des points de cassure ou éloigné de ses séquences régulatrices ou, comme dans les pathologies acquises, déplacé à côté d'un promoteur fort. Ces phénomènes peuvent donc être à l'origine d'une dérégulation transcriptionnelle et de l'altération du phénotype sans qu'il y ait de déséquilibre associé. Ces translocation « apparemment » équilibrées sont d'ailleurs depuis longtemps utilisées pour le clonage positionnel de gènes candidats responsables de divers syndromes ou maladies (Bugge *et al*, 2000 ; Johnson *et al*, 2006 ; Bache *et al*, 2006 ; Roohi *et al*, 2008).

Toutes ces études nous montrent l'intérêt d'une analyse pangénomique haute résolution en cas d'anomalie de structure apparemment équilibrée associée à un phénotype anormal. Malgré les difficultés d'interprétation des résultats obtenus par CGH sur puce rendant son utilisation délicate en diagnostic prénatal, il pourrait s'agir d'un outil précieux dans un contexte de remaniement de structure apparemment équilibré associé à des signes d'appel échographiques.

IV/ Anomalie chromosomique déséquilibrée à phénotype normal

A propos d'une délétion interstitielle du chromosome 11 (Goumy *et al*, 2008, Publication n°8)

Depuis longtemps il est établi que les anomalies chromosomiques constitutionnelles déséquilibrées concernant les régions euchromatiques des autosomes, qu'elles soient de nombre ou de structure, ont un retentissement phénotypique. Les manifestations cliniques sont liées à l'haploinsuffisance ou la triplosensibilité, c'est à dire à l'absence ou à la présence en trois exemplaires d'une des deux copies d'un ou plusieurs gènes localisés dans le segment délété ou dupliqué. Nous savons cependant que tous les gènes inclus dans la délétion/duplication ne sont pas nécessairement sensibles à cet effet de dosage génique. De rares cas de délétion/duplication de matériel euchromatique, parfois même de grande taille et visibles au caryotype standard et sans conséquence phénotypique, ont été décrits : ils sont appelés « variants euchromatiques » (Barber, 2005a ; Kolwalczyk *et al*, 2007).

Nous rapportons ici le cas d'un fœtus porteur d'une délétion interstitielle du bras long d'un chromosome 11 détectée au caryotype standard à partir d'une ponction de liquide amniotique réalisée pour signes d'appel biologique (marqueurs sériques de la

trisomie 21 à 1/170) chez une femme de 25 ans sans antécédents particulier. Les techniques moléculaires de cytogénétique (CGH sur métaphases et FISH avec des sondes locus spécifique commerciales et « à façon ») ont permis de préciser les points de cassure de cette délétion et le caryotype fœtal a été établi à 46,XX.del(11)(ql4.3q22.l). L'interrogatoire n'a pas révélé de notion de fausses couches à répétition ni de handicap dans la famille. L'enquête cytogénétique familiale a permis de retrouver la même délétion avec les mêmes points de cassure chez la mère et chez le grand père du fœtus. Le caractère hérité de l'anomalie, l'absence de répercussion phénotypique chez les ascendants ainsi que l'absence d'anomalie détectable aux échographies de contrôle ont permis d'être rassurant pour le conseil génétique et de proposer la poursuite de la grossesse. L'examen néonatal de l'enfant, né à terme, est sans particularité. Cette délétion familiale sans répercussion phénotypique est en faveur de l'existence d'une région haplo suffisante au niveau du bras long du chromosome 11, déjà évoquée en 2002 par Li *et al* (2002).

Notre cas a permis d'élargir la région chromosomique haplosuffisante de 3.6 Mb initialement décrite par Li à plus de 8,5 Mb. Les hypothèses avancées sont la faible densité en gènes de cette région et la compensation fonctionnelle par des gènes analogues situés ailleurs dans le génome. Ce cas a également permis d'exclue l'hypothèse d'une empreinte parentale faite par Li *et al* puisque la transmission de l'anomalie qu'elle soit paternelle ou maternelle n'entraîne pas de conséquence phénotypique. Enfin ce cas attire l'attention quant à la prudence à avoir pour le conseil génétique lors de la découverte en prénatal d'une délétion même visible au caryotype standard, tout particulièrement quand aucune répercussion phénotypique n'est constatée à l'échographie et l'importance de réaliser le caryotype des parents en première intention.

V/ Conclusion - Perspectives

Cette partie du travail montre la nécessité d'avoir recours aux techniques moléculaires de cytogénétique pour mieux appréhender les cas de discordances entre le caryotype et le phénotype observé. Il est ainsi possible : 1/ de mettre en évidence des anomalies cryptiques ; 2/ de mieux caractériser les anomalies en précisant les points de cassure et en étudiant la région chromosomique concernée (densité en gènes, nature des gènes, empreinte parentale…) ; 3/ d'éliminer la présence d'éventuels autres déséquilibres pouvant être à l'origine de l'altération du phénotype, ceci dans le but de mieux comprendre les mécanismes moléculaires impliqués.

Pour aller plus loin dans l'exploration de ces discordances, nous souhaitons poursuivre l'exploration de certains de ces patients à l'aide de puces pangénomiques haute densité. Nous envisageons également d'étudier, par diverses approches transcriptomiques, l'expression de certains gènes, comme par exemple ceux de la région 11q délétée et celle de *FLNB* dans le syndrome de Larsen atypique.

Publications n° 4 à 8

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SHORT COMMUNICATION

Prenatal detection of mosaic isochromosome 20q: a fourth report with abnormal phenotype

C. Goumy[1], A. M. Beaufrère[2], C. Francannet[3], A. Tchirkov[1], H. Laurichesse Delmas[4], F. Geissler[5], D. Lemery[4], P. J. Dechelotte[2] and P. Vago[1]*

[1]*Cytogénétique Médicale, CHU/Faculté de Médecine Clermont-Ferrand, France*
[2]*Anatomie et cytologie pathologiques, CHU Clermont-Ferrand, France*
[3]*Génétique Médicale, CHU Clermont-Ferrand, France*
[4]*Médecine Fœtale, CHU Clermont-Ferrand, France*
[5]*Service d'Imagerie Médicale, CHU Clermont-Ferrand, France*

We described a new case of mosaic isochromosome 20q revealed by amniocentesis. The propositus presented with craniofacial dysmorphism, clubfeet, and vertebral abnormalities. A 46,XX,i(20)(q10)[14]/46,XX[1] karyotype was confirmed by FISH on cultured cells. The pregnancy was terminated. From review of literature, fetus with mosaic isochromosome 20q identified on amniocentesis are most likely to be phenotypically and cytogenetically normal after birth. So we performed CGH and array-CGH to exclude another possible imbalance. We discuss here the possible relation between this chromosomal abnormality and the abnormal phenotype. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: isochromosome 20q; abnormal phenotype; prenatal diagnosis; genetic counselling

CASE REPORT

Amniocentesis was performed at 20 weeks of gestation because of abnormal sonographic examination with hydrocephaly, spinal malformation and bilateral clubfeet. The 27-year-old, gravida 2, para 0 (one termination of pregnancy) woman and her husband were healthy and non-consanguineous. There was no family history of congenital malformations. The pregnancy was terminated because of the multiple malformations. Post-mortem examination revealed a 245-g male fetus eutrophic, dysmorphic, with large mouth, flat little nose, hypertelorism, low set ears, large cervix with hygroma, bilateral club feet (Figure 1) and prominent sacral appendix. The brain examination showed hydrocephaly, major cerebellar hypoplasia and ocular anomalies (necrosis and calcification of the papilla). Radiography of the skull revealed dyssegmentation of the thoracic spine (Figure 2).

Figure 1—Post-mortem examination of the fetus showed bilateral clubfeet and craniofacial dysmorphism with hypertelorism, short and large neck, lowest ears

CYTOGENETIC STUDY

In 14 out of 15 separated colonies of amniocytes, an abnormal karyotype of 46,XY,i(20q) was noted. Only one colony had a 46,XY karyotype.

No post-abortion skin fibroblast karyotype was performed because of failure of fibroblasts culture.

FISH of interphase nuclei of amniocytes before culture using LSI 20q12 probe (VYSIS) revealed two signals in all of the 20 nuclei analysed. FISH of interphase nuclei of amniocyte after culture using the same probe showed three signals in 24% of the 260 nuclei analysed.

CGH on chromosome spread was performed, as described previously (Goumy *et al*., 2005), to exclude another genomic imbalance. DNA was extracted from cultured amniocytes. This technique allows a whole genome investigation with a resolution above 5 Mb. No imbalance was found.

Then, in order to increase resolution, we performed array-CGH using GenoSensor Array 300 (Abbott). This array investigates 287 loci with a resolution of 50 to 200 kb. DNA was extracted from cultured amniocytes,

*Correspondence to: P. Vago, Cytogénétique Médicale, Faculté de Médecine, BP 38, 63001 Clermont-Ferrand Cedex, France.
E-mail: carole.goumy@u-clermont1.fr

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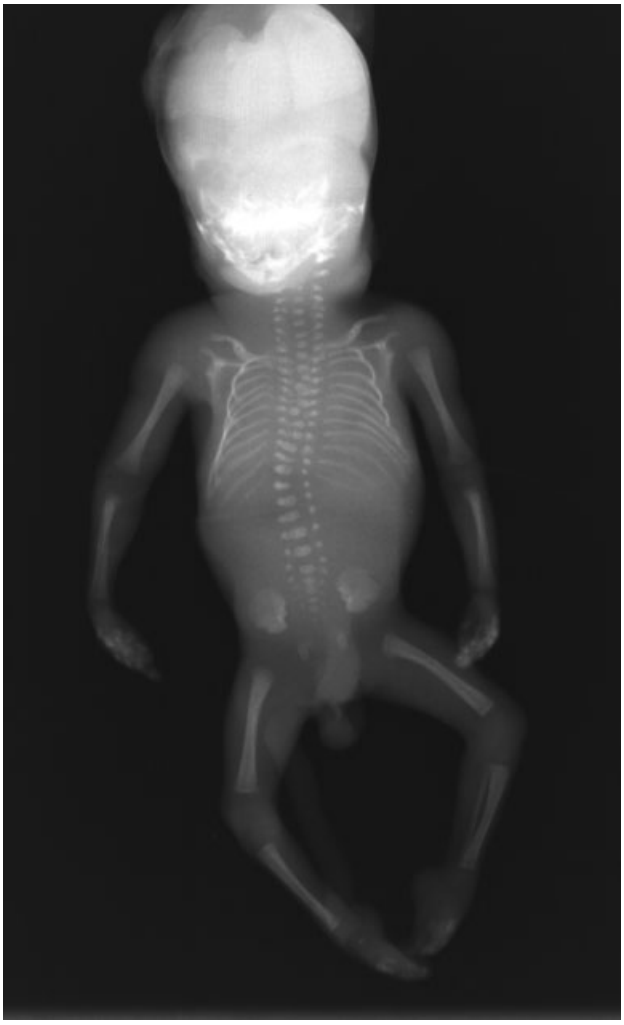

Figure 2—Radiography of the skull showed dyssegmentation of the thoracic spine and clubfeet

and the technique was performed according to manufacturer recommendations. The 287 clinically relevant genomic clones spotted on this array include all the telomeres, as well as all genome region implicated in the known microdeletion syndromes and additional selected loci representing each chromosome arm.

As for CGH, neither chromosome 20 imbalances (any gain of 20q or loss of 20p) nor another imbalance elsewhere in the genome were found.

DISCUSSION

To our knowledge, this is the first case of i(20q) investigated using a FISH technique performed on uncultured amniocytes and using CGH and array-CGH for detecting another genomic imbalance.

In Pfeiffer's study (1997), FISH of interphase nuclei of buccal epithelium revealed the presence of an isochromosome 20q in only 6% of the cells. This rate is at the limit of the detection of this technique, which is of 5% in our laboratory. In our case, the absence of abnormal cells in the direct preparation and their presence in cultured cells suggest that the cell culturing has contributed to the proliferation of abnormal cells. The percentage of abnormal cells in the primary sample was probably weak and the number of nuclei analysed (20) insufficient to detect the mosaicism by FISH in the direct preparation. Nevertheless, our fetus had an abnormal phenotype.

CGH on chromosome spread was performed to exclude the possibility of another genomic imbalance elsewhere in the whole genome, with a resolution above 5 Mb. To detect an eventual micro-imbalance (<5 Mb), we performed CGH array using an array containing 287 clinically relevant clones. Although the whole genome was not explored, the main regions implicated in known rearrangements were investigated, with a resolution of 50 to 200 kb.

As expected, neither chromosome 20 short arm loss nor chromosome 20 long arm gain have been detected by CGH or array-CGH. This could be explained by the fact that these two techniques are unable to detect weak mosaicisms. On the other hand, using these two techniques, we have excluded another imbalance which could explain the abnormal phenotype. Obviously, a micro-imbalance <5 Mb located outside the 287 clinically relevant loci investigated by the array or a gene disruption could not be excluded.

In the large majority of cases, the phenotype of the fetus and the subsequently born child with i(20q) mosaicism and its psychomotor development are completely normal (for review see Chen, 2003). Discussions of the published prenatal observations concurred with the conclusion that the cell line with this chromosomal abnormality might be confined to extraembryonic tissues and that a normal outcome of these pregnancies could be expected. In most cases, the mosaicism of i(20q) is limited to the amniocytes (for review see Chen, 2003). Rare exceptions were the following: Chernos *et al*. (1992) reported a case with diaphragmatic hernia, ventricular dilatation, anophthalmia, and craniofacial dysmorphism. Pfeiffer *et al*. (1997) reported a newborn with scars on the scalp, hypoplasia of the *corpus callosum*, right microphthalmos, orbital cyst, deformed thoracal vertebral body, rocker-bottom feet and normal psychomotor development at 7 months of age. Chen (2003) reported a case with arthrogryposis multiplex congenital and amyoplasia. In these three reports, the authors concluded that a casual relationship between the abnormal phenotype and the i(20q) is uncertain. The pattern of malformations described in these three reports does not point to a known specific syndrome but in Chernos' and Pfeiffer's cases, as in our case, the clinical features evoked suspicion of a chromosomal abnormality. Some phenotypic features of our fetus are found in these two reports. Chernos *et al* reported on a hydrocephaly and Pfeiffer *et al* on ocular abnormalities, clubfeet, and exactly the same spinal malformations. Moreover, vertebral anomalies are frequently associated with chromosome 20 short arm deletions (Kalousek and Thérien, 1976; Kogame *et al*., 1978; Garcia-Cruz *et al*., 1985; Vianna-Morgante *et al*., 1987; Kiss and Osztovics, 1988; Silengo *et al*., 1988; Dutta *et al*., 1991) and microphtalmy, low set ears, clubfeet and cerebellar atrophy are described in cases

with a chromosome 20 long arm duplication (Sax *et al*., 1986; Pierquin *et al*., 1988; Herens *et al*., 1990; Plotner *et al*., 2002).

All together, these findings, particularly the absence of imbalance detected by CGH and array-CGH and the similarity of the clinical feature with Pfeiffer's report, concurred with the conclusion that the abnormal phenotype is probably linked to the mosaicism of i(20q). Interestingly, i(20q) might be unstable, leading to wide variability in different tissues and at different periods of development; this could explain the other cases that were reported to be normal.

We conclude that cases of prenatal detection of i(20q) mosaicism, even if the rate of abnormal cells is weak, should be taken with caution and the genetic counselling should be done carefully on the basis of a referent ultrasonographic investigation searching for vertebral dyssegmentation and cerebral malformations.

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Larsen-like phenotype associated with partial trisomy 3p and monosomy 5p

C. Goumy[1], A. M. Beaufrère[2], A. Tchirkov[1], L. Gouas[1], F. Gaspard[3], M. Giollant[1], T. Roucaute[1], L. Veronèse[1], D. Lemery[4] and P. Vago[1]*

[1]*Univ Clermont1, UFR Médecine, CHU Clermont-Ferrand, Service de Cytogénétique Médicale, France*
[2]*CHU Clermont-Ferrand, Service d'Anatomie Pathologique, Hôtel-Dieu, France*
[3]*CHU Clermont-Ferrand, Service d'Imagerie Médicale, Hôtel-Dieu, France*
[4]*CHU Clermont-Ferrand, Service de Gynécologie Obstétrique, Maternité, Hôtel-Dieu, France*

Background We report on a fetus with radiographic features of Larsen Syndrome (LS) and unbalanced 3;5 translocation. Recently LS was shown to be caused by mutations in *FLNB* gene which maps on 3p14.3.

Methods Comparative genomic hybridization (CGH) was performed to search for genomic imbalances. Fluorescence in situ analysis (FISH) was done with BAC clone RP11-754F19 probe from the *FLNB* gene region (3p14.3).

Results CGH showed a large loss of the chromosome 5 short arm and a gain of half of the short arm of chromosome 3 resulting from a derivative chromosome 5. FISH analysis with *FLNB* probe demonstrated that it was not triplicated. Thus, we excluded the role of a gene dosage effect of *FLNB* in abnormal craniofacial development in this fetus.

Conclusions To our knowledge, this is the first report of Larsen-like phenotype associated with unbalanced translocation resulting in partial trisomy 3p and monosomy 5p.

KEY WORDS: Larsen-like syndrome; trisomy 3p/monosomy 5p; CGH

INTRODUCTION

Larsen syndrome (LS; OMIM 150 250 and 245 600) is characterized by multiple congenital joint dislocations and craniofacial abnormalities (Larsen *et al*., 1950). Other abnormalities including clubfoot, cylindrical fingers, cleft palate, and spinal abnormalities have been described. Some familial cases have been described, with both autosomal dominant and recessive patterns of inheritance and extreme variability in clinical presentation (Becker *et al*., 2000). Recently LS was shown to be caused by mutations in the *FLNB* gene which maps on 3p14.3 (Krakow *et al*., 2004; Zhang *et al*., 2006; Bicknell *et al*., 2007). Here we report on clinical findings and molecular cytogenetic studies in a fetus with a 46,XY,der(5)t(3;5)(p21;p13) detected by CGH (comparative genomic hybridization) and radiological features suggestive of LS.

CASE REPORT

A 23-year-old prima gravidia was referred for amniocentesis at 21 weeks of gestation because of abnormal sonographic examination with spina bifida, 'lemon and banana signs', oligoamnios, cystic renal dysplasia considered to be a caudal regression syndrome. Nuchal translucency was 1.1 mm at 10 weeks of gestation. The parents were counseled and they opted for pregnancy termination. Postmortem examination revealed hypertelorism, down-turned mouth, low-set and malformed ears, short neck, micrognathia, prominent forehead, flat occiput and flattened face, fingers abnormalities, fifth finger clinodactyly, genu recurvatum, large lumbar spina bifida *aperta*, bilateral clubfoot, heart defect (interventricular communication), weak pulmonary hypoplasia, and multicystic bilateral renal dysplasia not compatible with life (Figure 1a).

Radiological examination of the skeleton revealed complete and bilateral dislocation of the tibia on the femur, unilateral dislocation of the hip joint, deformation of lumbar vertebrae associated with the spina bifida, bilateral clubfoot, flat faces, and ossification defect of the pubis (Figure 1b).

Mother family history reveals recurrent miscarriage and the aunt of the mother had a fetus with polymalformation who died *in utero* at 8 months and a girl who died 5 days after birth and presented facial dysmorphy (hypertelorism, microretrognathism, hypoplasia at the base of the nose), posterior cleft palate, bilateral single transverse palmar crease and thumb folded across palm, agenesis of the corpus callosum, and tetralogy of Fallot.

CYTOGENETIC STUDY

Method

Karyotyping was performed on amniocyte and skin fibroblast cultures with RHG and GTG-banding according to standard methods.

*Correspondence to: P. Vago, Cytogénétique Médicale, Faculté de Médecine, BP 38, 63001 Clermont-Ferrand Cedex, France.
E-mail: pvago@chu-clermontferrand.fr

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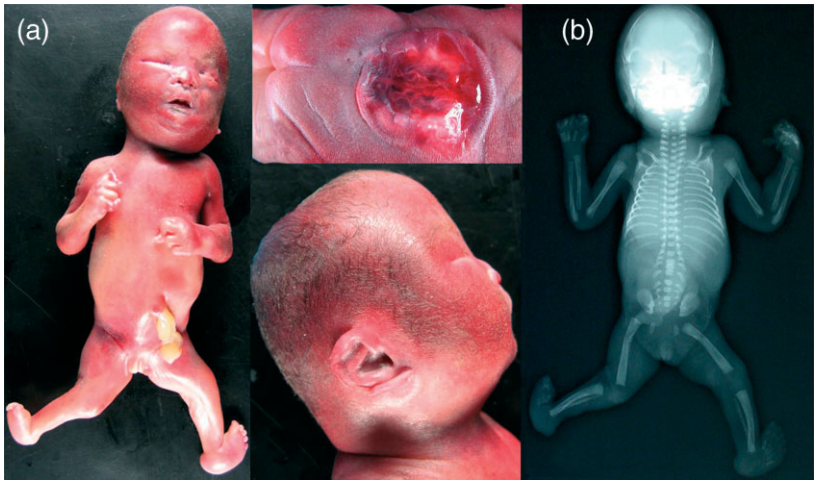

Figure 1—Postmortem examination of the fetus: (a) external aspect of the fetus revealed dysmorphic face with hypertelorism, low-set and malformed ears, short neck, micrognathia, prominent forehead, flat occiput, and flattened face; fingers abnormalities, fifth finger clinodactyly, genu recurvatum, bilateral clubfoot, and large lumbar spina bifida aperta. (b) Radiography of the skull showed complete and bilateral dislocation of the tibia on the femur, unilateral dislocation of the hip joint, deformation of lumbar vertebrae associated with the spina bifida, bilateral clubfoot, and ossification defect of the pubis suggestive of Larsen syndrome

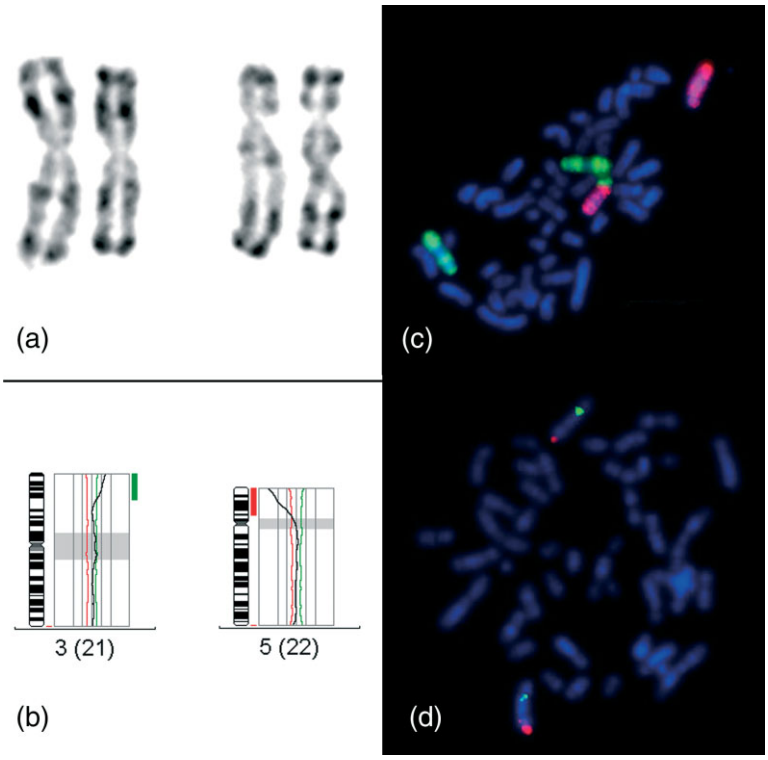

Figure 2—Cytogenetic investigations (a) partial karyotype of R-banded chromosomes 3 and 5, (b) CGH profile of chromosomes 3 and 5 showed an amplification 3p21→pter and a deletion 5p13→pter, (c) FISH analysis of metaphase chromosomes using WCP 3 (green) and WCP 5 (red) confirmed the presence of a derivative chromosome 5, (d) FISH with the probe including *FLNB* (green) and a subtelomeric 3q control probe (red) showed two signals for *FLNB* on both normal chromosomes 3

CGH was performed on chromosome spread using DNA extracted from cultured amniocytes, as described previously (Goumy *et al*., 2005).

Fluorescence *in situ* hybridization (FISH) analysis was carried out on metaphase spreads with WCP 3 and WCP 5 probes (Abbott) and BAC clone RP11-754F19 probe (Invitrogen) for the 3p14.3 region that includes the *FLNB* gene, according to the manufacturer's instructions.

Results

The karyotype 46,XX was considered as normal (Figure 2a). CGH on metaphase showed a 3p21→pter gain

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and a 5p13→pter loss (Figure 2b). FISH with WCP 3 and WCP 5 confirmed the rearrangement with the presence of a derivative chromosome 5 (Figure 2c). FISH with the probe including the *FLNB* gene showed two signals on both normal chromosomes 3 (Figure 2d). FISH analysis in the mother revealed the balanced t(3;5)(p21;p13).

DISCUSSION

There is increasing evidence that cytogenetically invisible chromosome rearrangements are an important cause of genetic diseases. In the present study, standard karyotype was considered as normal. Because of the large number of malformations in this fetus we performed CGH which revealed a large deletion of the chromosome 5 short arm and an amplification of half of the short arm of chromosome 3. This imbalance resulted from a balanced maternal t(3;5)(p21;p13). Despite the large size of the rearrangement the karyotype failed to detect the translocation in either the infant or his mother because of the likeness between the two exchanged chromosomal segments. This case indicates that some chromosomal rearrangements, even if they are >10 Mb, are nonapparent and shows the superiority of the CGH compared with karyotype, not because of a higher resolution but because a more objective nonoperator dependent interpretation.

The clinical features of the monosomy 5p ('Cri du Chat' syndrome) are well known for a long time (Lejeune *et al*., 1963). The syndrome is characterized in young children by microcephaly, round face, hypertelorism, micrognathia, epicanthal folds, low-set ears, hypotonia, and severe psychomotor and mental retardation. The most characteristic feature in newborn children is a high-pitched cat-like cry that is usually considered diagnostic for the syndrome (Church *et al*., 1995).

The phenotype of partial trisomy 3p is also very characteristic with a specific chromosomal aberration syndrome proposed by Gimelli (Gimelli *et al*., 1985). Clinical characteristics present in this syndrome are psychomotor and mental retardation, square face, short neck, dysplasic ears, hypertelorism, and micrognatia. In addition to dysmorphic stigmata, cerebral malformations (microcephaly, hydrocephaly, or holoprosencephaly), and congenital heart defect have been described (Reiss *et al*., 1986; Gillerot *et al*., 1987; Conte *et al*., 1995). Witters *et al*. (2004) report a prenatal case at 12 weeks of gestation with multiple congenital anomalies including micrognathia, cleft palate, low-set ears, enlarged posterior fossa, bilateral pes equinovarus, anal atresia, ambiguous external genitalia, internal genitalia with absent uterus and left gonad (chromosomal sex XY), tetralogy of Fallot, cystic renal dysplasia, and a single umbilical artery.

To date, only one article reports a similar unbalanced translocation with trisomy 3p23→pter and monosomy 5p14→pter (Entezami *et al*., 1997). In this article, the sonographic examination showed a single umbilical artery, polyhydramnios, and nondemonstrability of the stomach. Postmortem examination revealed atresia of the oesophagus, hypertelorism, depressed nasal bridge, anteverted nostrils, and large dysplasic ears. Cranial sonography showed partial holoprosencephaly and a pathological gyration pattern. No joint dislocation was described (Table 1).

LS is a skeletal dysplasia with multiple joint dislocations and craniofacial abnormalities. In our case, radiographic signs were suggestive of LS, namely, flat face, complete and bilateral dislocation of the tibia on

Table 1—Comparison of clinical manifestations

	Partial monosomy 5p [a]	Partial trisomy 3p[b]	Tri 3p23→pter and mono 5p14→pter[c]	Larsen syndrome	Present case
Holoprosencephaly	−	+	+	−	−
Microcephaly	+	+	−	−	−
Flat occiput	−	−	−	+	+
Hypertelorism	+	+	+	+	+
Micrognathia	+	+		−	+
Flat nose	−	+	+	+	+
Epicanthus	+	+		−	−
Low−set/malformed ears	+	+	+	−	+
Cleft palate	−	+	−	Inconsistent	−
Neck	−	Short	−	−	Large
Face	Round	Square	−	Square and flat	Square and flat
Frontal bossing	−	+	−	+	+
Heart defect	−	+	−	Inconsistent (IVC[d])	IVC[d]
Spina bifida	−		−	Inconsistent	+
Cystic renal dysplasia	−	+	−	−	+
Gonadal dysgenesis	−	+	−	−	−
Single umbilical artery	−	+	+	−	−
Clubfoot	−	−	−	+	+
Joint dislocations	−	−	−	+	Knee, left hip

[a] Lejeune *et al*., 1963; Church *et al*., 1995.
[b] Gimelli *et al*., 1985; Reiss *et al*., 1986; Gillerot *et al*., 1987; Conte *et al*., 1995; Witters *et al*., 2004.
[c] Entezami *et al*., 1997.
[d] Interventricular communication.

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the femur, unilateral dislocation of the hip joint, and club foot deformity (Table 1). The anomaly of the lower limbs could not be explained by the oligoamnios, which was moderate, but club foot might be a consequence of the spina bifida.

Recently, LS was shown to be caused by missense mutations or small in-frame deletions in *FLNB*, encoding the cytoskeletal protein filamin B (Bicknell *et al*., 2007). Curiously, the breakpoint on chromosome 3 is located near the *FLNB* gene that maps in 3p14.3. To verify in our case whether *FLNB* was triplicated, FISH analysis was made with a BAC clone covering the gene region. *FLNB* was present in only two copies suggesting that *FLNB* dosage effect did not determine the LS phenotype in this fetus.

Previously only three cases reported a Larsen-like syndrome associated with unbalanced chromosomal translocation resulting in partial 1q (two cases) or 10q trisomy and distal 6p monosomy (Pierquin *et al*., 1991; James *et al*., 2003). To our knowledge, this is the first report of Larsen-like syndrome associated with trisomy 3p and monosomy 5p. This case reflects the importance of chromosomal studies even if clinical diagnosis is evident for an accurate genetic counseling.

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ARTICLE ORIGINAL

AN UNUSUAL FAMILIAL CHROMOSOME 9 "VARIANT" WITH VARIABLE PHENOTYPE: CHARACTERIZATION BY CGH ANALYSIS

C. GOUMY [1], M. MIHAESCU [1], A. TCHIRKOV [1], M. GIOLLANT [1], M.N. BONNET-DUPEYRON [1], J.Y. JAFFRAY [1], A. GENEIX [1], B. PERISSEL [1], C. FRANCANNET [2], O. BOESPFLUG-TANGUY [2], P. VAGO [1]

(1) Cytogénétique Médicale, CHU et Faculté de Médecine, BP 38, 63001 Clermont-Ferrand Cedex.

(2) Génétique Médicale, CHU et Faculté de Médecine, BP 69, 63003 Clermont-Ferrand Cedex.

SUMMARY

Heterochromatin confined to pericentromeric and secondary constriction regions plays a major role in morphological variation of chromosome 9, because of its size and affinity for pericentric inversion. We report on a 6-year-old boy with growth and language delay, minor facial anomalies and unusual chromosome 9 variant with an extra-band in the centromeric region on the conventional karyotype. Subsequent analysis by FISH and CGH identified this variant as a dicentric chromosome 9 with a duplication of the 9p12-q21 region. An identical chromosome 9 variant was found in the mild language retarded brother and in the phenotypically normal father and grandfather. The presumed mechanism accounting for the phenotypic discordance observed in this family and the usefulness of CGH in characterization of such variants are discussed. To our knowledge, this is the first investigation of an unusual chromosome 9 variant by CGH.

Key words: chromosome 9 variant. dicentric. phenotypic variability. duplication. CGH.

RÉSUMÉ

Caractérisation par CGH d'un variant familial inhabituel du chromosome 9 avec variabilité phénotypique

Les variations de taille de la constriction secondaire et les inversions péricentriques du chromosome 9 limitées à l'hétérochromatine sont fréquentes. Nous rapportons le cas d'un garçon âgé de 6 ans qui présentait un retard de croissance et de langage, une dysmorphie faciale mineure et un variant inhabituel du chromosome 9 avec une bande supplémentaire localisée dans la région centromérique sur le caryotype standard. Les études plus poussées par FISH et CGH ont permis d'identifier ce variant comme un chromosome 9 dicentrique avec une duplication de la région 9p12-q21. Le même variant a été retrouvé chez le frère présentant un retard de langage modéré ainsi que chez le père et le grand-père, phénotypiquement normaux. Le mécanisme probable rendant compte des discordances phénotypiques dans cette famille et l'utilité de la CGH sont discutés. À notre connaissance, il s'agit de la première analyse par CGH d'un tel variant inhabituel du chromosome 9.

Mots-clés : *variant du chromosome 9. dicentrique. variabilité phénotypique. duplication. CGH.*

INTRODUCTION

The heterochromatic secondary constriction region of chromosome 9 is one of the most variable in the human karyotype: 9qh+ and inv(9)(p11q13), with frequencies of approximately 8% and 1.5% respectively, are very common findings in routine cytogenetics [15, 22, 35, 36]. These frequent structural alterations are regarded as normal familial variants, which are termed heteromorphism and inherited in a Mendelian fashion without any apparent phenotypic consequences [1, 2]. Previous studies attributed the extensive degree of variability in this region to unequal meiotic exchanges involving the various classes of repetitive sequences in the centromeric region and to unequal exanges involving stretches of homologous DNA sequences in the proximal short and long arms [32]. According to Stark *et al.* [32], homologies between 9p12 and 9q13-21.1 regions are observed, and these regions are also described homologous to the short arms of the acrocentric chromosomes.

However, there are few reports of variants involving euchromatin. We describe here a case of unusual familial chromosome 9 "variant" showing a duplication of the pericentromeric p12q21 region with phenotypical variability.

CLINICAL REPORT

A 6-year-old boy was referred for diagnostic evaluation because of his minor facial anomalies and psychomotor and growth retardation. He was born to a healthy 30-year-old mother at term after a non complicated pregnancy. The Apgar score was 8/9, the birth

Correspondence: P. VAGO, address above.
E-mail: pvago@chu-clermontferrand.fr

weight was 3,320 g, the length was 50 cm and the head circumference was 35 cm. He was slightly delayed in development in that he sat at 9 month and started to walk at 18 month. He spoke some words at 2 years. On physical examination, his height was 105 cm (25th centile), his weight was 17 kg and OFC was 52.5 cm, the both on the 50th centile. He displayed minimal dysmorphic craniofacial features with a high forehead, a wide glabella, a bulbous nose, a short philtrum, a large mouth (+ 3DS) *(figure 1)*. The ears were large (+1DS) with posterior ear lobes grooves. Hyperconvex nails and a clinodactyly of the fifth fingers were noticed.

He has one older brother (9 years) with a mild dysphasia. The father and the grandfather were not dysmorphic and displayed no obvious evidence of learning difficulties. Except these two children, family history was unremarkable.

METHODS

Routine cytogenetic studies

Karyotyping was performed on peripheral blood lymphocytes according to the standard procedures using RHG and GTG banding techniques.

Fluorescence In Situ Hybridization (FISH)

FISH analysis was carried out with whole chromosome painting 9 probe (WCP 9), chromosome 9 enumeration probe (CEP 9) and telomere 9q probe (TEL 9q) purchased from Vysis [ABBOTT, Rungis, France], on metaphase chromosomes and interphase nuclei according to the manufacturer's instructions. Images were captured using a CCD-camera attached to a Zeiss Axioplan microscope.

Comparative Genomic Hybridization (CGH)

CGH analysis was performed as described previously [9] with minor modifications. Patient and reference DNAs was extracted using DNAzol reagent (Invitrogen, Cergy Pontoise, France). Patient DNA (test) and normal male DNA (reference) were labelled with dUTP FITC and dUTP Texas red respectively using standard nick translation reaction. Both labelled DNAs and unlabelled Cot-1 DNA (Vysis) were cohybridized to normal male metaphase spreads for 3 days at 37° in humid chamber. Then slides were washed once in 0.4xSSC for 2 minutes and in 2xSSC for 30 seconds at room temperature. They were counterstained with DAPI II (Vysis). CGH slides were analysed through a Zeiss Axioplan epifluorescence motorized microscope equipped with a cooled CCD camera (Hamamatsu) connected to a PC computer which include Isis software (Metasystems). Grey levels digital pictures were captured separately for each fluorochrome: DAPI (blue), FITC (green) and Texas Red (red). At least 10 metaphases were analysed. Karyotyping was performed based on DAPI-banding pattern. Green and red fluorescence intensities were measured classically along the length of each chromosome. Ratio-profiles-fluorescence (RPF) were calculated for each chromosome. Chromosomal regions with a RPF above 1.25 were considered to be over-represented (gain), whereas chromosome regions with a RPF below 0.8 were considered to be under-represented (loss).

RESULTS

Analysed of G and R-banded metaphases from the proband revealed the presence of an extra-band within the elongated centromeric region *(figure 2a)*. Chromosomal analysis in the brother, the father and the grandfather showed a similar unusual chromosome 9 *(figure 3)*. Whole chromosome 9 specific painting probe did hybridize to the extra-band *(figure 2b)*. FISH with CEP9 probe showed the presence of two signals on the rearranged chromosome 9 *(figure 2c)*. By CGH, a gain of the segment 9p12q21 was observed in the proband *(figure 2d)*, the brother and the father *(figure 3)*. Because the structural/repetitive DNA is excluded from the CGH analysis (blocked by Cot-1 DNA), the CGH result indicates that genes (euchromatin), with a potentially dose dependant influence on phenotype, might be present in this duplicated material.

The combination of conventional and molecular cytogenetics allowed us to describe the karyotype for the proband, his brother, his father and his grandfather as follows: 46,XY,dup(9)(pter->q21::p12->qter).

DISCUSSION

Chromosome 9 exhibits a wide spectrum of features that range from the common heterochromatin variants to the rare euchromatic variants with an extra-band in 9qh+ or 9ph+. There are some reports of such rare chromosome 9 variants segregating in families without any phenotypic effect [4, 6-8, 10, 22, 31-34, 37, 38].

In the present observation, the "variant" is a dicentric chromosome 9 with duplication of the segment 9p12-q21 as delineated using CGH. CGH analysis gives a global overview of chromosomal gains and losses throughout the whole genome [24, 30]. CGH analysis is able to determine the euchromatic nature of chromosomal extra-bands because the structural/repetitive DNA is blocked by Cot-1 DNA and excluded from analysis. The resolution of CGH is 5-10 Mb, comparable with that of high resolution karyotype but with a more objective approach. In our case, CGH analysis showed that the extra-band was constituted by chromosome 9 material, thus confirming the euchromatic nature of the extra-bands and, in the limit of its resolution, excluding the presence of other unbalanced chromosomal anomalies.

According to Macera [21] and Lukusa [20], this rearrangement could have arisen by an unequal crossing-over during the first meiotic division, because of sequence homology at the breakpoints of the rearrangement [32]. This supposes chromatid breaks at 9p12 in

FIG. 1. — Patient photographs.

FIG. 1. — *Photographies du patient.*

one chromosome and at 9q21 in the homologous chromosome, followed by rejoining of the broken ends of the centromere bearing chromatids of the two homologs.

In 7 cases reported by Starke et al [32], the chromosome 9 variants were detected in children or young adults in connection with psychomotor retardation. There are also several reports of variant bands in the 9qh associated with spontaneous abortions [28] or abnormal phenotype: hydrocephaly and spina bifida as the fetal ultrasound [13], small weight and atrial septal defect [26], hypoplasic lungs and foetal hydrops [17], mental retardation and minor facial anomalies [20]. In all these cases, with similar 9qh regions involved but

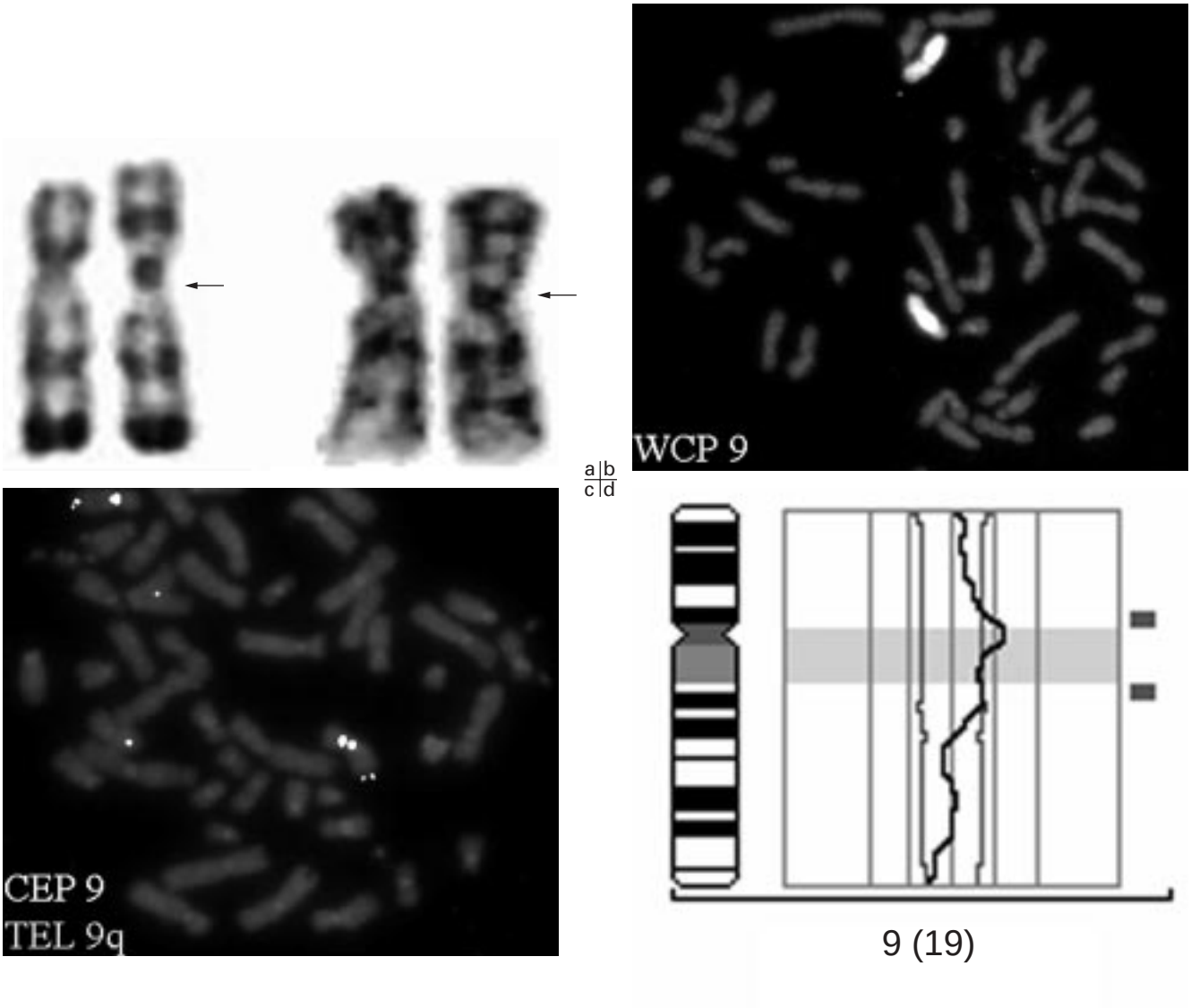

FIG. 2. — Cytogenetic investigations in the propositus. a) Partial karyotype of R and G-banded chromosomes, showing the normal chromosome 9 and the "variant" chromosome 9, arrows indicate the additional material present in the proximal short arm; b) chromosomes 9 stained by FISH technique using WCP probe; c) FISH analysis of metaphase chromosomes with CEP 9 and TEL 9q probes; d) CGH profil of chromosome 9.

FIG. 2. — *Investigations cytogénétiques du propositus. a) caryotype partiel en bandes R et G montrant le chromosome 9 normal et le variant. Les flèches indiquent le matériel excédentaire présent au niveau du bras court du chromosome. b) chromosomes 9 colorés par la technique de FISH avec une sonde WCP. c) Analyse en FISH des chromosomes en métaphase avec les sondes CEP 9 et TEL 9q. d) Profil en CGH du chromosome 9.*

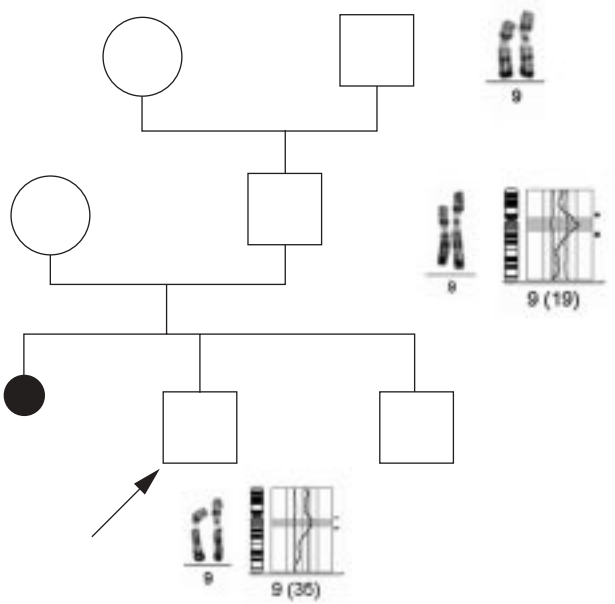

FIG. 3. — Truncated pedigree and partial cytogenetic analysis of the family with R-banded chromosomes 9 and CGH profil in the brother and the father.

FIG. 3. — *Arbre généalogique partiel et analyse cytogénétique partielle de la famille avec les chromosomes 9 marqués en bandes R et le profil en CGH du frère et du père.*

different clinical manifestations, the authors conclude that the chromosome 9 variant appears to be unrelated to the patient's phenotype because a similar chromosome was found in one of their phenotypic normal parent. It should be noted that none of these cases have been investigated by CGH which could have detected an additional rearrangement.

In our case, the same chromosome 9 unusual pattern is present in 3 generations with variable phenotype. The presence of similar chromosomal abnormality in the same family with discordant phenotypical characteristic is intriguing but not uncommon. For example, the 22q11 deletion syndrome shows wide spectrum of phenotypes although most patients have a common microdeletion [40]. In the same way, Reddy et Fugate [27] reported a proband and father who both had trisomy 5p15.3 and monosomy 18p11.32 but a different phenotype and Baker et al [3] described a subtelomeric deletion 12p with variable phenotypic effect in a boy and his mother.

Several hypotheses to explain the phenotypic variability have been proposed.

1. Chromosome 9 variant is unrelated to the patient's phenotype which could depend on another undetected imbalance.

2. The index patient could have inherited an additional cryptic rearrangement (deletion or amplification) at the site of the breakpoints [11, 12, 25]. Such mechanism could explain the phenotypic abnormalities in cases of apparently "balanced" translocation [16, 19]. Indeed, the rearrangement might facilitate an unequal crossing-over during meiosis, resulting in submicroscopic imbalance.

3. In our case, in the phenotypically normal subject, the reason for the silencing of the genes in the euchromatin band could also be due to position effect: the euchromatic band sandwiched between heterochromatic blocs might have been more or less inactivated [14, 37]. This mechanism is similar to the variable inactivation spreading in case of X-autosome translocation: the spreading of inactivation into autosomal sequences may be incomplete and discontinuous [5, 18, 29].

Finally, clinical expression could also be controlled by non genetic factors, which modulate the phenotype.

In the present study, CGH allows to exclude an additional unbalanced rearrangement. Moreover it also allows to precise that the extra-band comprise euchromatin from 9p12q21 region. As there are phenotypic normal parents in the family, such an isolated amplification could be *a priori* considered as harmless. Howerver, additional cryptic rearrangements or silencing variability could be responsible for the abnormal phenotype observed in the patient.

In conclusion, our report demonstrates that CGH analysis is useful in investigating cases with unusual chromosome 9 variant, particularly in de novo cases, to verify if the gain of material on the chromosome 9 correspond to the region 9p12q21. Such information is valuable for genetic counselling. To our knowledge, our report is the first study using CGH for characterization of such unusual variant.

ACKNOWLEDGEMENTS

We thank the patient family for participation and Dr Jean-Michel Dupont for critical review of the manuscript.

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DE NOVO BALANCED COMPLEX CHROMOSOME REARRANGEMENT (CCR) INVOLVING CHROMOSOME 8, 11 AND 16 IN A BOY WITH MILD DEVELOPMENTAL DELAY AND PSYCHOTIC DISORDER

BY C. GOUMY [1], M. MIHAESCU [1], A. TCHIRKOV [1], M. GIOLLANT [1], C. BENIER[1], C. FRANCANNET [2], J.Y. JAFFRAY [1], A. GENEIX [1] AND P. VAGO [1]

Summary: *De novo balanced complex chromosome rearrangement (CCR) involving chromosome 8, 11 and 16 in a boy with mild developmental delay and psychotic disorder*: Congenital Complex Chromosome rearrangements (CCRs) compatible with life are rare in humans. We report a de novo CCR involving chromosomes 8, 11 and 16 with 4 breakpoints in a patient with mild dysmorphic features, acquisition delay and psychotic disorder. Conventional cytogenetic analysis revealed an apparently balanced 8;16 translocation. Further FISH analysis with WCP 8 and WCP 16 probes revealed the presence of a third chromosome involved in the translocation. The multicolour karyotype confirmed the complexity of the rearrangement and showed that the derivative chromosome 8 was composed of 3 distinct segments derived from chromosomes 8, 16 and 11. The breakpoints of this complex rearrangement were located at 8q21, 11q14, 11q23 and 16q12. Comparative genomic hybridization (CGH) and array-CGH were performed to investigate the possibility of any genomic imbalance as a result of the complex rearrangement. No imbalance was detected by these two techniques. Our study showed: i) the necessity to confirm reciprocal translocations with FISH using painting probes, particularly when the karyotype resolution is weak; ii) the usefulness of multicolour karyotype for the characterization of structural chromosomal rearrangements, particularly when they are complex; iii) the usefulness of CGH and array-CGH in cases of abnormal phenotype and apparently balanced rearrangement in order to explore the breakpoints and to detect additional imbalances.

Key-Words: CCR - M-FISH – CGH - Developmental delay - Psychotic disorder.

INTRODUCTION

Complex chromosomal rearrangements (CCRs) are rare structural chromosome aberrations characterized by three or more breakpoints located on two or more chromosomes (17). More than 130 constitutional CCRs have been documented (for review see 2, 13, 14, 16, 22). M-FISH has proven to be a powerful tool for characterization of these types of rearrangements. In patients with abnormal phenotype and apparently balanced chromosomal rearrangement, even simple reciprocal translocation, submicroscopic deletion/duplication could be present in the regions of breakpoints or elsewhere. Patients with balanced complex rearrangements are at even higher risk of carrying such submicroscopic rearrangement. Therefore, at least a high resolution karyotype should be performed. Today, an alternative approach

(1) Cytogénétique Médicale, CHU / Faculté de Médecine, Clermont-Ferrand, France.

(2) Génétique Médicale, CHU Clermont-Ferrand, France.

by comparative genomic hybridization (CGH) can be proposed. CGH analysis allows a genome-wide screen for chromosomal gains or losses; however, its resolution is usually limited to 5-10 Mb (3). It is also possible to perform array-CGH which reveals cryptic gains and losses with a higher resolution (1).
Here, we report one case of de novo CCR involving chromosomes 8, 11 and 16 occurring in an 11 year old boy with principally a psychiatric development disorder. This CCR involved 3 chromosomes with 4 breakpoints, 2 of which occurred on the long arm of chromosome 11. We present the results of molecular cytogenetic investigations, which were performed to characterize such CCR and to investigate a possible imbalance.

CLINICAL FINDINGS

The patient was born at term to healthy, non consanguineous parents. The family history was unremarkable; the patient has a normal younger sister. The mother has no history of exposure to known mutagens, drugs, radiation or viral infections before or during pregnancy. Birth weight was 3,180 g, length 50 cm, and head circumference 35 cm. No neonatal problems were reported except bilateral testicular ectopy. The Apgar score was 10/10. He sat alone at 6 month and started walking at 14 month.
He was referred at the age of 11 years for analysis of sex chromosomes because of tall and slender morphologic aspect, learning disabilities and behaviour problems such as shyness and immaturity.
Physical examination revealed a height of 153 cm (+2 DS), weight of 33 kg (+1 DS) and normal head circumference (51.5 cm). He had minor facial dysmorphism with slight hypotelorism, hypoplastic alae nasi with septum extending below nasal alae, appearance of a relatively large mouth compared to other facial structure, absence of one inferior canine and ogival palate (Fig. 1).

Figure 1: Patient photographs

Psychiatric evaluation showed psychiatric disorder with poor social interaction, stereotyped interest and anxiety. He has slight acquisition delay and difficulties in immediate memory and in temporal location. The EEG was normal.

MATERIALS AND METHODS

Conventional Cytogenetic

Karyotyping was performed on lymphocytes cultures from peripheral blood with RHG-banding according to standard methods.

Fluorescence In Situ Hybridization

FISH analysis was carried out with whole chromosome painting 8 and 16 probes (WCP 8 and WCP 16) purchased from Vysis [Abbott, Rungis, France], on metaphase spreads and according to the manufacturer's instructions. Images were captured using a CCD-camera attached to a Zeiss Axioplan microscope (Carl Zeiss Jena GmbH, Jena, Germany).

M-FISH

The "24 XCyte" M-FISH probe kit and software were applied (MetaSystems, Altlussheim, Germany). A Zeiss Axioplan epifluorescence microscope was used for capturing images.

Comparative Genomic Hybridation (CGH)

CGH was performed as described previously (6) with minor modifications. Patient and reference DNAs was extracted using DNAzol reagent (Invitrogen, Cergy Pontoise, France). Patient DNA (test) and normal male DNA (reference) were labelled with dUTP FITC and dUTP Texas red respectively using standard nick translation reaction. Both labelled DNAs and unlabelled Cot-1 DNA (Vysis) were hybridized to normal male metaphase spreads for 3 days at 37° in humid chamber. They were counterstained with DAPI II (Vysis). CGH slides were analysed through a Zeiss Axioplan epifluorescence motorized microscope equipped with a cooled CCD camera (Hamamatsu) connected to a PC computer which include Isis software (Metasystems). Green and red fluorescence intensities were measured classically along the length of each chromosome. Ratio-profiles-fluorescence (RPF) were calculated for each chromosome. Chromosomal regions with a RPF above 1.25 were considered to be over-represented (gain), whereas chromosome

regions with a RPF below 0.8 were considered to be under-represented (loss).

Array-CGH

The commercially array used (GenoSensor Array 300) contains 287 genomic clones, including those for each human telomeres, as well as all of the known microdeletion syndromes and additional selected loci representing each chromosome arm. Each clone is represented by three target spots.
Test and reference DNA was prepared for hybridization, according to manufacturer recommendations, with the GenoSensor Array 300 Kit (Abbott). The reference DNA had the same sex of test DNA. The array was imaged and data analysed using the GenoSensor Reader and its accompanying software (Vysis/Abbott).

RESULTS

Analysis of RHG-banded metaphases from the patient revealed the presence of an apparently balanced reciprocal translocation between chromosomes 8 and 16 (Fig. 2). FISH with WCP 8 and WCP 16 probes indicated that a third chromosome was involved in the rearrangement

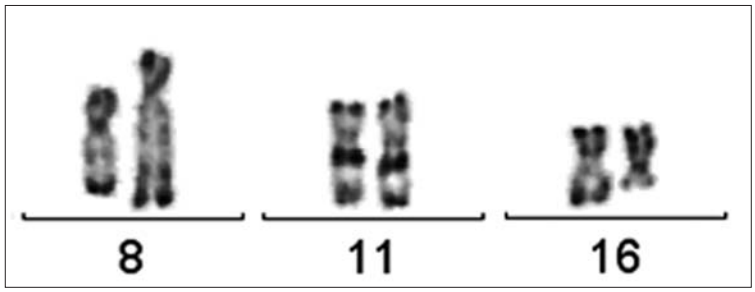

Figure 2: RHG banded karyotype showing an apparently balanced reciprocal translocation between chromosomes 8 and 16.

(Fig. 3): the derivative chromosome 16 was not stained by WCP 8 probe but bore genetic material from another chromosome and WCP8 probe stained another unidentified derivative chromosome.
M-FISH confirmed the complex nature of the karyotype showing the presence of three rearranged chromosomes: derivative chromosomes 8, 11 and 16 (Fig. 4). These investigations allowed establishing that the rearrangement involved 3 chromosomes with 4 breakpoints: one on derivative chromosome 8 assigned to 8q21, two on derivative chromosome 11 assigned to 11q14 and 11q23 and one

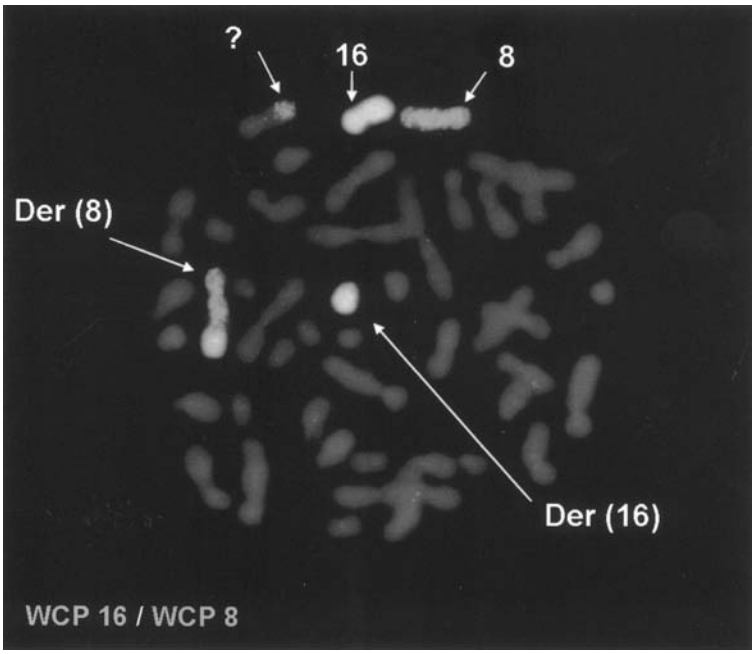

Figure 3: FISH using WCP 8 and WCP 16 probes showed normal chromosomes 8 and 16, the derivative chromosome 8 stained by WCP 8 and WCP 16 probes, the derivative chromosome 16 partially stained by WCP 16 probe and a third derivative chromosome partially stained by WCP 8 probes

on derivative chromosome 16 assigned to 16q12. So the rearranged chromosomes were: der(8)(8pter→8q21::11q14→11q23::16q12→16qter), der(11)(11pter→11q14::8q21→8qter); der(16)(16pter→16q12::11q23→11qter).

CGH analysis showed no imbalance at the level neither of the 4 breakpoints nor elsewhere in the genome (Fig. 5). Array-CGH also did not revealed any imbalance at the 287 investigated loci, including the 11q13-q14 (clones GARP, PAK1), 11q23 (clone MLL) and 16q12-q13 (clone CYLD1) regions.

Standard karyotypes of both the parents and the sister of the index case were normal.

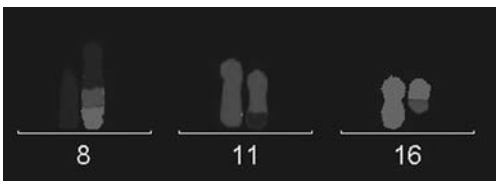

Figure 4: M-FISH emphasised that three chromosomes were rearranged: a chromosome 8 composed of 3 distinct segments derived from chromosomes 8, 11 and 16; a chromosome 11 bearing material from a chromosome 8 and 11; a chromosome 16 stained by chromosome 11 and chromosome 16 probes.

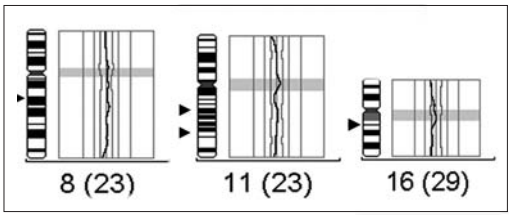

Figure 5: CGH results for chromosomes involved in the translocation. Translocation breakpoints are indicated on the chromosome ideograms (arrows).

DISCUSSION

We describe here the combined application of FISH, M-FISH, CGH and array-CGH in the study of a de novo CCR associated to a mild developmental delay and psychiatric disorder. Routine cytogenetic investigations are often insufficient to elucidate CCRs. Moreover this case emphasizes the necessity to investigate apparently simple reciprocal translocations by FISH at least with WCP probes particularly in case of weak resolution karyotype. M-FISH appears relevant for more accurate characterization of CCRs at the cytogenetic level but has limits (~10Mb) in detecting intra-chromosomal rearrangements (12).

Three major categories of CCRs were described by Gardner and Sutherland (5): the most common is the three-way translocation; the second is a coincidence of two separate simple reciprocal translocations; the third includes more complicated CCR with more than one breakpoint per chromosome. Our case belongs to the third category: 3 chromosomes involved with 4 breakpoints.

Lurie *et al.* (15) studied the probability of breakpoint recurrent involvement in 33 CCR cases without any primary intrachromosomal abnormalities such as inversion, insertion, or duplication. The authors found that chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12 and 21 were involved in CCRs with chromosomes 3 and 7 being the most frequent. They propose the possible explanation that the initial event leads to an unstable provisional rearrangement, and subsequent breaks are necessary to stabilize the karyotype. In our case, the chromosome 8 breakpoint located at 8q21, the two chromosome 11 breakpoints at 11q14 and 11q23, and the chromosome 16 breakpoint at 16q12. The complexity of the present CCR resides in the fact that 2 of 4 breakpoints were within the long arm of chromosome 11. The chromosome 16 implication in a CCR is rare: in Batanian and Eswara study (2), only five cases out of 100 have been reported to show breakpoints at 16p11 (twice), 16p13, 16q13 and 16q22. Nevertheless in a more recent study 6 CCRs out of 27 (~ 20%) have a breakpoint at chromosome 16 at 16p13, 16q12 (twice) and 16q22 (three times) (22). Thus far, it is only the third report of a CCR involving the 16q12 breakpoint. On the other hand, chromosomes 8 and 11 are frequently involved in CCR, 8q21, 11q14 and 11q23 being recurrent breakpoints (2).

Generally, de novo CCRs, even when balanced, are associated with mental retardation, congenital abnormalities and reproductive problems (4, 7, 8, 9, 17, 18, 19). In a large study by Warburton (24), serious malformations were found in 6.1% of pregnancies with a de novo reciprocal translocation. According to Madan *et al.* (16), the risk of phenotypic abnormality in balanced CCRs is greater than the 4-6%

risk associated with apparently balanced reciprocal translocations. Lespinasse *et al*. speculate on a 3.5% risk per breakpoint to calculate a cumulative risk (13). In most patients balanced CCRs are assumed to be the cause of the observed phenotypic abnormalities because of submicroscopic deletions, duplications or disruption of genes located at the breakpoints. So far, this hypothesis has rarely been proven by molecular analysis. In a recent case, a cryptic 7q31.3 deletion has been found elsewhere that in breakpoints using array-CGH in an apparently balanced complex translocation involving chromosomes 5, 6 and 7 (21).

In our report, the phenotype of the patient was abnormal with slight dysmorphic features, psychotic disorders and acquisition delay. This phenotype with de novo CCR suggested some submicroscopic imbalance and led to specific cytogenetic investigations such as CGH (resolution ~5 Mb) and array-CGH to prove or exclude the presence of cryptic rearrangement, in breakpoint as in Lespinasse report (14) or elsewhere in the genome as in the case of Tyson report (21). In our case these investigations showed no imbalance, but the array used (GenoSensor array 300) contains a limited number (287) of known clinically relevant clones. The detection capabilities of array-CGH are limited by the format of the array being used. A higher resolution could be obtained with a "pangenome" 1 Mb resolution array (23). To our knowledge, this is the fourth report which used GenoSensor Array 300 to explore mental retardation, malformations and/or spontaneous miscarriages (10, 11, 20). As Schaeffer *et al.*, we think that this technique is more reproducible and the interpretation more easily than pangenome array CGH.

Finally, it is also possible that a gene disruption at one of the breakpoints could be responsible for the psychiatric disorder. The likelihood of such event increases with increasing number of chromosomal breaks. A molecular analysis of the 4 breakpoint regions would be required in order to assess this hypothesis.

In conclusion, our report showed that the number of breakpoints and the complexity of the CCRs are generally underestimated. We advise then to verify the reciprocal translocations by FISH or by performing high resolution karyotype. In case of CCRs, a more accurate description can be obtained using M-FISH. Finally, when the translocation appeared balanced and the phenotype is abnormal, CGH and array-CGH are relevant for emphasising or excluding imbalances, within the breakpoint regions or elsewhere.

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ADDRESS FOR CORRESPONDENCE:

Professeur Philippe VAGO
Service de Cytogénétique Médicale
Faculté de Médecine – BP 38
63001 Clermont-Ferrand Cedex, France
Tel: +33 (0)4 73 17 81 00; Fax: +33 (0)4 73 26 91 82
E-mail: carole.goumy@u-clermont1.fr

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Clinical Report

Familial Deletion 11q14.3–q22.1 Without Apparent Phenotypic Consequences: A Haplosufficient 8.5 Mb Region

C. Goumy,[1] L. Gouas,[1] A. Tchirkov,[1] T. Roucaute,[1] M. Giollant,[1] L. Veronèse,[1] C. Francannet,[2] and P. Vago[1]*

[1]Univ Clermont1, UFR Médecine, CHU Clermont-Ferrand, Service de Cytogénétique Médicale, France
[2]CHU Clermont-Ferrand, Service de Génétique Médicale, Hôtel-Dieu, France

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We present the prenatal diagnosis of a chromosome 11q14.3–q22.1 deletion identified in three generations without apparent phenotypic consequences. A 25-year-old G2, P1 woman underwent amniocentesis at 15 weeks' gestation because of a positive result for Down syndrome maternal serum-screening test (1/70). The fetal karyotype revealed an interstitial deletion of the long arm of chromosome 11 confirmed by CGH and FISH: 46,XX,del(11)(q14.3q22.1). The mother and grandfather of the fetus presented the same interstitial deletion with a little if any phenotype effect. The pregnancy was carried to term and resulted in the birth of a normal girl. To our knowledge, only one case of a chromosome 11q14.3–q21 deletion without phenotypic anomalies has been reported. Our study allows the initially described haplosufficient region to be extended from 3.6 Mb to at least 8.5 Mb. This large deletion was compatible with fertility and apparently normal phenotype. Identification of such chromosomal regions is important for prenatal diagnosis and genetic counseling. © 2008 Wiley-Liss, Inc.

Key words: haplosufficiency; 11q14.3–q22.1 deletion; transmitted imbalance; normal phenotype

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INTRODUCTION

Cytogenetically detectable deletions of euchromatic segments are generally expected to result in an abnormal phenotype. There is, however, a small group of several chromosomal regions that upon deletion do not seem to cause an abnormal phenotype [Barber, 2005; Kowalczyk et al., 2007]. Identification of such deletions is essential for prenatal diagnosis and genetic counseling. Here we report on the second-trimester prenatal diagnosis of an interstitial 11q14.3–q22.1 deletion with direct transmission in three generations without apparent phenotypic effect. The complete characterization of the abnormality by CGH and FISH as well as the control analysis of parental chromosomes were performed. Finally, we consider the possible contributory factors which might explain the lack of clinical significance of this large deletion.

CLINICAL REPORT AND CYTOGENETIC STUDY

The patient was a G2P1 25-year-old woman, who had amniocentesis because of a positive Down syndrome maternal serum-screening result at 15 weeks' gestation. Ultrasound scans at 11 and 15 weeks gestation were normal. The family history was unremarkable.

Chromosome analysis was performed on amniocyte cultures with GTG-banding according to standard methods and revealed a deletion of the long arm of chromosome 11 (Fig. 1a).

Molecular Cytogenetic Analysis

Comparative genomic hybridization (CGH) was performed on chromosome spreads using DNA extracted from cultured amniocytes, as previously described [du Manoir et al., 1995; Goumy et al., 2005],

*Correspondence to: Prof. P. Vago, Cytogénétique Médicale, Faculté de Médecine, BP 38, 63001 Clermont-Ferrand Cedex, France.
E-mail: pvago@chu-clermontferrand.fr

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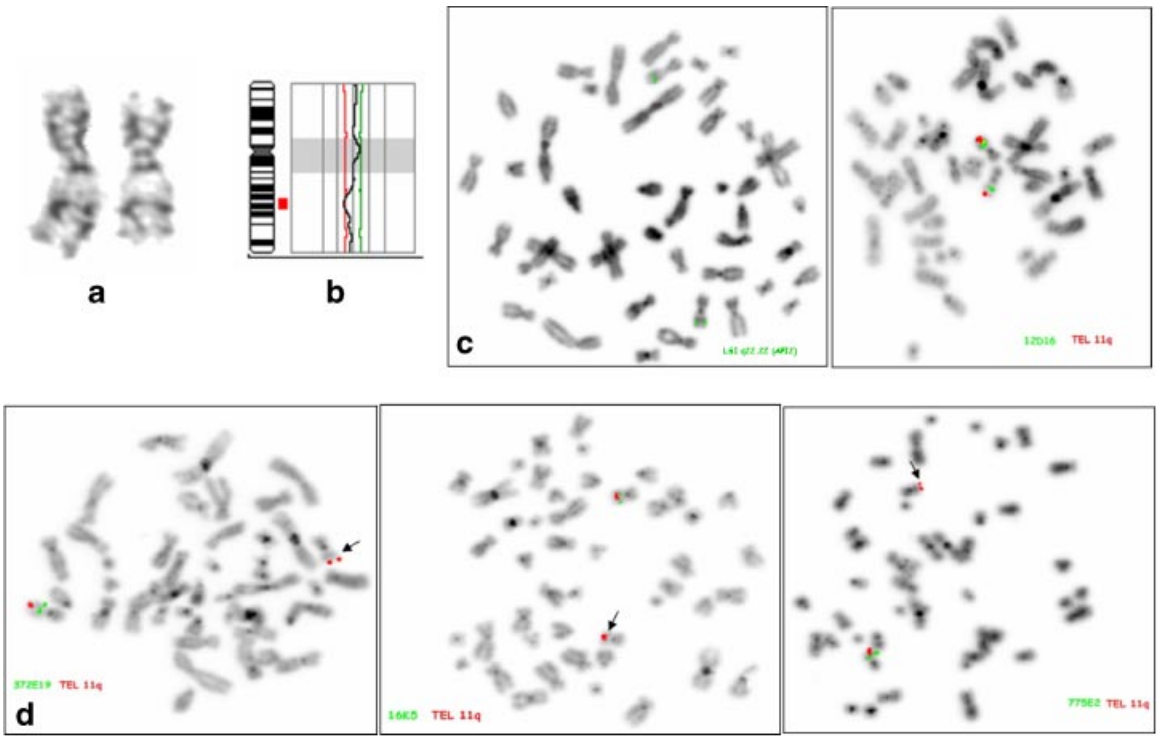

FIG. 1. **a**: Partial GTG-banded karyotype of the fetus illustrating normal (left) and deleted (right) chromosome 11 homologues. **b**: CGH profile of chromosome 11 showing the 11q14.3 → 11q22.1 deletion. **c**: FISH with the Vysis API2 probe localized at 11q22.22 and a BAC clone RP11-12D16 localized at 11q14.2 showed two green signals. **d**: FISH with BAC clones mapping to 11q14.3 (RP11-372E19), 11q21 (RP11-16K5), and 11q22.1 (RP11-775E2) showed only a single copy of these probes (arrows). The subtelomeric 11q probe (Vysis) was used as a control (red signals). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with a resolution of 3–5 Mb determined in our laboratory. The CGH confirmed the 11q deletion and mapped the breakpoints at 11q14.3 and 11q22.1 (Fig. 1b). FISH (fluorescence in situ hybridization) experiments were performed on metaphase spreads with a commercial probe (LSI API2, Vysis) according to the manufacturer's instructions, and with bacterial artificial chromosome (BAC) selected from the human library RPCI-11 according to the UCSC Human Genome Assembly (March 2006). BAC DNA extraction was done using the NucleoSpin® Plasmid kit (Macherey-Nagel GmbH, Hoerdt, France). BAC DNAs were then labeled with Fluorescein-12-dUTP (Roche Diagnostics GmbH, Meylan, France) by nick translation (Abbott Molecular, Rungis, France).

FISH results with a BAC clone localized at 11q14.2 (RP11-12D16) showed two signals, confirming a breakpoint location between 11q14.2 and 11q14.3, whereas the commercial probe LSI API2 at 11q21 also showed two signals which was not concordant with the 11q22.1 breakpoint shown by CGH (Fig. 1c). FISH with BAC clones localized at 11q14.3 (RP11-372E19), 11q21 (RP11-16K5), 11q22.1 (RP11-775E2) showed one signal on one of the chromosome 11 homologues (Fig. 1d) and made it possible to confirm that the deletion extended to the 11q22.1 band. Using the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu) with the last genome assembly (NCBI Build 36.1, March 2006), we were able to replace the position of the commercial probe LSI API2 from 11q21, as given by the manufacturer, to 11q22.2. This new localization explained the presence of two FISH signals with this probe and confirmed the breakpoint in 11q22.1.

By combining karyotype, CGH and FISH results, the karyotype was described as follows: 46,XX.ish del(11)(q14.3q22.1)(RP11-372E19-,RP11-16K5-,RP11-775E2-). The minimal deletion size estimated as the distance between the proximal edge of BAC RP11-372E19 and the distal edge of BAC RP11-775E2 was 8.5 Mb and the maximal deleted size evaluated between the distal edge of RP11-12D16 and the proximal edge of the LSI API2 probe was 16 Mb (Fig. 2).

Familial Study

Chromosome analyses were performed on both parents who apparently were phenotypically normal. The same interstitial deletion was observed in the mothers' karyotype. CGH analysis showed the same profile with breakpoints at 11q14.3 and 11q22.1 and similar FISH results were obtained with the BAC clones used in the fetus (data not shown). The mother's clinical examination and interrogation

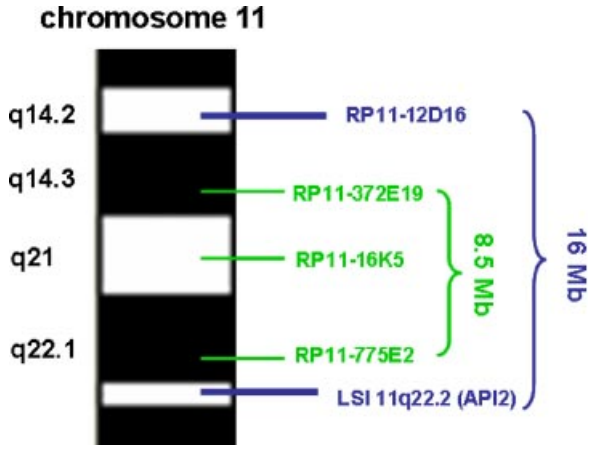

FIG. 2. Partial chromosome 11 ideogram showing the BAC clones used to define the deletion. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

revealed endometrial polyps (which were treated by hysteroscopic surgery), toe camptodactyly and ophtalmologic disorders (hypermetropia, astigmatism, strabismus). There was no evidence of multiple miscarriages, birth defects or developmental delay in the mother's family.

The maternal grandparents were then explored, and cytogenetic analysis in the phenotypically normal grandfather revealed the 11q14.3q22.1 deletion (data not shown).

Pregnancy Issue

The genetic counseling was reassuring. The pregnancy was carried to term and resulted in the birth of a normal girl without dysmorphic features. Birth weight was 3,400 g, length 50 cm and head circumference 35 cm. The Apgar scores were 8/8/10 and clinical examination was normal.

DISCUSSION

This case provides further evidence that rare unbalanced deletions of specific chromosomal regions may result in the absence of phenotypic effect and underlines the importance of obtaining parental karyotypes before making a clinical decision. The interstitial deletion 11q14.3–q22.1 described here was identified in three individuals from three generations without apparent phenotypic consequences. The camptodactyly of the toes and ophthalmological features observed in the mother were not present in the grandfather. These features are common in the general population and are unlikely to be related to the deletion.

In 2007, Kowalczyk et al. reviewed cases of euchromatic chromosome imbalances, mainly inherited, without phenotypic abnormalities. They found 15 chromosomal regions on chromosomes 2, 3, 5, 7, 8, 9, 10, 11, 13, 16, and 18 for which deletions are observed in phenotypically normal individuals. Some of these, namely deletions 5p14 [Overhauser et al., 1986; Johnson et al., 2000], 10q11 [Bisgaard et al., 2007], 16q21 [Hand et al., 2000], and 2p12 [Barber, 2005] have each been found in more than one independent family and transmitted without consistent phenotypic consequences. In the review by Barber [2005], the average size of such deletions was 8.2 Mb (range 4.2–16 Mb) and these deletions generally involved G dark bands to which few genes map.

To our knowledge, only one asymptomatic deletion of the long arm of chromosome 11 has been described [Li et al., 2002]. This report identified a 3.6 Mb deletion at 11q14.3–q21 in five individuals representing three generations of kindred. Only one carrier showed mild clinical features, which were probably unrelated to the deletion.

In our case, the deletion was visible on standard karyotype with a resolution of about 10 Mb. FISH with commercial and BAC probes mapped the breakpoints at 11q14.3 and 11q22.1 and made it possible to estimate the size of the deletion between 8.5 and 16 Mb. Thus, our report enables the haplosufficient region initially described by Li et al. to be extended from 3.6 Mb to at least 8.5 Mb. This 8.5 Mb region contains only 22 protein coding genes (Table I; http://www.ncbi.nlm.nih.gov/mapview).

Stratton et al. [1994] described a chromosome11q14.1–q21 deletion in a 4-year-old girl with moderate developmental delay, horseshoe kidney, bilateral duplication of the ureters with right upper pole obstruction, hydronephrosis and Wilms tumor. In this case, the 11q14.3–q21 region of deletion overlaps with the haplosufficient region described here, suggesting that the genes responsible for the abnormal phenotype may be localized between 11q14.1 and 11q14.3.

Horelli-Kuitunen et al. [1999] described a de novo deletion of 11q21–q22.3 in a 3-year-old girl with disproportionate short stature, hypotonia with "myopatic changes" at the muscle biopsy, developmental delay, hypertelorism, low-set ears, pes equinovarus of the right foot, mild hydronephrosis, enlarged cerebral lateral ventricles and thin corpus callosum. Meyer et al. [2000] reported a 11q21–q23.1 deletion in a 21-year-old woman associated with motor and speech developmental delay in infancy, congenital heart defect, craniofacial anomalies, menstrual irregularity, hirsutism, elevated serum androgen levels and polycystic ovary syndrome. Likewise, Li et al. [2006] reported an 11q14.1–q23.2 deletion and predicted that the distal segment 11q21–q23.3 may be responsible for the varying degrees of growth retardation, mental retardation, cleft palate and minor digital anomalies of the patient. Assuming that the 11q14.3–q22.1 region might be haplosufficient, the regions responsible for the abnormal

TABLE I. Genes Mapping Within the Minimal Deletion Size (chr11: 91,373,125–99,929,275*)

Gene symbol	Gene name	Expression	Gene/protein family	Function	Related disease	Other gene family location
FAT3*	Tumor suppressor homolog 3 (drosophila)	ES cells, primitive neuroectoderm, fetal brain, infant brain, adult neural tissues and prostate	Cadherin superfamily members	Cell adhesion	None	FAT1 at 4q35, FAT2 at 5q32q33, FAT4 at 4q28.1
MTNR1B[a]/MEL-1B-R	Melatonin receptor 1B	Retina, brain	Receptor for melatonin	Receptor for melatonin	600804	MTNR1A at 4q35.1
SLC36A4/PAT4	Solute carrier family 36	Unknown	Amino acid transporter genes	Proton-coupled transporters	None	Cluster at 5q33.1
JOSD3/MGC5306	Josephin domain containing 3	Only in human carcinoma and tumor cell lines	Unknown	Repair pathway in carcinogenesis	None	22q13.1 19q13.33
MED17/CRSP6/ TRAP80	Mediator complex subunit 17/Cofactor required for Sp1	Ubiquitously	Transcription cofactor complex CRSP	Cofactor required for transcription initiation by the RNA pol II	603810	Multiple
HEPHL1	Hephaestin-like	Unknown	Unknown	Bind copper Feroxydase activity Intestinal iron absorption	None	Unknown
PANX1/MRS1	Pannexin 1	Ubiquitously abundantly in several brain regions and retina	Innexin/pannexin superfamily	Structural components of gap junctions	608420	PANX2: 22q13.33 PANX3: 11q24.2
GPR83/GIR	G protein coupled receptor 83	Brain	G protein-coupled receptors	Unknown	605569	2p14
MRE11A	Meiotic recombination 11 homolog A	Ubiquitously High level in proliferating tissues	MRE11 family	Homologous recombination, telomere length maintenance, DNA DSBs repair	600814	MRE11B at 3q25
ANKRD49/FGIF	Ankyrin repeat domain 49	Unknown	Unknown	Unknown	None	Unknown
FUT4	Fucosyltransferase 4	Embryos (5–10 weeks)	Fucosyltransferases	Biosynthesis of Lewis antigene	104230	Multiple FUT9: 6q16
PIWIL4	Piwi-like 4 (drosophile)	Adult testis	PIWI-like family	Development and maintenance of germline stem cells	610315	12q24.33, 8p21.3, 22q11.23
AMOTL1	Angiomotin like 1	Endothelial cells of capillaries and placenta vessels	AMOTL2	Structural component of tight junctions	None	3q21q22
JMJD2D	Jumonji domain containing 2D	Prostate	JMJD2 family	Histone demethylase/Androgen receptor activators	609766	1p34.1, 9p24.1, 19p13.3
SFRS2B/SRP46	Splicing factor, arginine/ serine-rich 2B	Multiple tissues	Serine/arginine-rich family	Pre-mRNA Splicing factor	603269	Multiple
ENDOD1	Endonuclease domain containing 1	Unknown	Unknown	Unknown	None	Unknown
SESN3	Sestrin 3	Unknown	Sestrin family/ PA26-related gene family	Unknown	607768	1p35.3 6q21
CEP57/PIG8	Centrosomal protein 57kDa/ translokin	Ubiquitinously	Unknown	Mediates FGF2 nuclear translocation and mitogenic activity	607951	Unknown
MTMR2/CMT4B	Myotubularin related protein 2	Ubiquitinously	Myotubularin related family (14 members)	Tyrosine phosphatase	603557	Xq28, 13q12
MAML2/MAM2	Mastermind-like2		Mastermind proteins family	NOTCH coactivator signaling pathway	607537	5q35, 4q28
JRKL/HHMJG	Jerky homolog-like (mouse)	Ubiquitinously	Unknown	Nuclear regulatory protein	603211	Unknown
CNTN5/NB-2	Contactin 5	Developing nervous system, brain moderate in thyroid and placenta	Immunoglobulin superfamily	Cell adhesion molecule	607219	12q11q12, 1q32.1, cluster at 3p26

*From Human Genome Assembly Build 36 (March 2006).
[a]FAT1-MTNR1A locus at 4q35.2 and FAT3-MTNR1B locus at 11q14.3–q21 were paralogous regions (Katoh and Katoh, 2006).

phenotype in these three cases could be reduced to 11q22.1–q22.3, 11q22.1–q23.1, and 11q22.1–q23.2, respectively.

The hypotheses proposed to explain the lack of clinical consequences in case of such large deletions were the low density of genes in the deleted region, the haplosufficiency of the majority of these genes or the presence of genes with similar function located elsewhere in the genome and a possible genomic imprinting effect leading to the loss of inactive material [Li et al., 2002; Barber, 2005].

Our observation does not support the genomic imprinting hypothesis [Bortotto et al., 1990; Li et al., 2002] because the transmission of the deletion in three generations of apparently normal individuals was of both paternal and maternal origin, even if the psychomotor development of the newborn is difficult to assess.

The majority of the deleted genes have related loci on other chromosomes, suggesting that functional complementation can arise from isoform expression or that alternative metabolic pathways can substitute those affected (Table I). The other genes are probably not dosage sensitive.

Recently, identification of inherited and de novo imbalances of uncertain clinical significance has progressively escalated with the increasing use of array technology in diagnostic laboratories. Knowledge of such harmless imbalances is particularly helpful for prenatal diagnosis and genetic counseling.

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3ème partie

3ème PARTIE : Modèle de la hernie diaphragmatique congénitale (HDC) : une altération du phénotype associée à diverses anomalies chromosomiques

I/ Introduction

La hernie diaphragmatique congénitale (HDC) est une malformation sévère, très hétérogène phénotypiquement et génotypiquement. Malgré les acquisitions récentes sur la pathogénie et la physiopathologie de la HDC, le diagnostic anténatal et les progrès de la réanimation néonatale et de la chirurgie, le pronostic de cette malformation n'a pas fondamentalement changé.

La physiopathologie des HDC est encore incomplètement élucidée mais de nombreux travaux ont montré qu'il existait un lien entre la signalisation des rétinoïdes et la HDC (Greer *et al*, 2003 ; Gallot *et al*, 2005).

Les HDC peuvent être isolées ou associées à d'autres malformations, souvent pulmonaires et cardiaques, et peuvent alors faire partie de syndromes plus ou moins connus ou être associées à une anomalie chromosomique.

Une partie de ce travail a consisté à répertorier les anomalies chromosomiques décrites dans la littérature chez des fœtus ou patients porteurs de HDC et rechercher dans les régions chromosomiques remaniées des gènes candidats impliqués directement ou indirectement dans la voie de signalisation des rétinoïdes. Ce travail nous a permis d'identifier de nombreux gènes candidats et de discuter de leur possible implication dans la genèse des HDC.

Nous avons parallèlement étudié l'expression de plusieurs acteurs du signal rétinoïque dans des fibroblastes cutanés de fœtus porteurs ou non de HDC.

La finalité de ces travaux était d'étayer l'hypothèse de l'implication des rétinoïdes dans la genèse des hernies diaphragmatiques et d'identifier de nouveaux gènes candidats.

II/ Etude bibliographique

1) La Hernie Diaphragmatique Congénitale

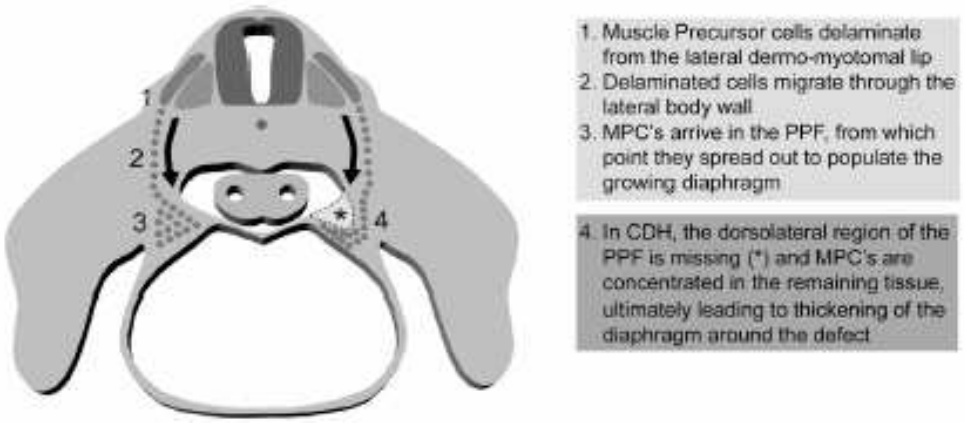
La hernie diaphragmatique congénitale (OMIM 142340, 222400, 610187 et 306950) se définit comme l'absence de développement de tout ou partie du diaphragme laissant persister une communication entre les cavités pleurale et péritonéale. En raison de

la pression différente entre l'abdomen et le thorax, les organes abdominaux se déplacent dans le thorax entraînant une hypoplasie pulmonaire et une hypertension artérielle pulmonaire responsables d'un taux de mortalité élevé (30 à 60%) par détresse respiratoire néonatale (Harrison *et al*, 1994 ; Nobuhara *et al*, 1996**)**. Cette pathologie survient dans environ une naissance sur 3 000 (Langham *et al*, 1996 ; Torfs *et al*, 1992 ; Skari *et al*, 2000). Plus de 90% des HDC sont postéro-latérales (hernie de Bochdalek) et parmi elles, 85% sont à gauche, 10% à droite et 5% sont bilatérales (Barker *et al*, 1993 ; Hedrick *et al*, 2004). Le diagnostic se fait le plus souvent dans la période prénatale, dès 20 SA si il s'agit d'une forme grave. Le taux de détection échographique de cette pathologie en France est compris entre 50 et 60% (Gallot *et al*, 2007).

Il s'agit d'une malformation précoce puisque le développement du diaphragme intervient entre la 4ème et la 12ème semaine de gestation. Les hypothèses, sur les évènements embryonnaires conduisant à la formation des HDC, sont encore controversées. Les études les plus récentes suggèrent que la HDC serait due à un défaut de développement des membranes pleuropéritonéales touchant le compartiment mésenchymateux et non le compartiment musculaire (Fig. 5) (Gosche *et al*, 2005 ; Clugston *et al*, 2006 ; Clugston et Greer, 2007). D'autres hypothèses ont été émises, telles qu'une colonisation musculaire insuffisante ou un défaut de colonisation par le nerf phrénique (Allan et Greer, 1997), sans qu'aucune ne soit retenue de façon unanime (Thebaud *et al*, 1999 ; Babiuk et Greer, 2002 et Babiuk *et al*, 2003).

FIGURE 5 : Modèle de développement de la HDC de type Bochdalek
d'après Clugston *et al*, 2007

Coupe transversale au niveau de la région cervicale d'un embryon de rat au stade E13



Dans près de la moitié des cas, les HDC sont associées à d'autres malformations ce qui laisse supposer qu'il s'agit souvent de la manifestation d'une embryopathie globale dont l'origine serait une dérégulation de mécanismes cellulaires fondamentaux lors de l'organogénèse (Bielinska *et al*, 2007). Les malformations associées sont le plus souvent des cardiopathies, des anomalies du système nerveux central, des fentes labio-palatines et des anomalies rénales et squelettiques (Dott *et al*, 2003 ; Tonks *et al*, 2004 ; Bedoyan *et al*, 2004 ; Graham et Devine, 2005 ; Gallot *et al*, 2007). Ces hernies non isolées peuvent rentrer dans le cadre d'un syndrome génétique ou peuvent être associées à une anomalie chromosomique (Skari *et al*, 2000 ; Witters *et al*, 2001 ; Slavotinek, 2005a ; Graziano, 2005 ; Holder *et al*, 2007 ; Scott, 2007a).

2) Les HDC « syndromiques »

La fréquence des HDC entrant dans le cadre de syndromes cliniques a été estimée, selon les études, entre 14 et 44% (Cunniff *et al*, 1993 ; Enns *et al*, 1998). Les HDC sont décrites dans plus de 70 syndromes différents. Le syndrome de Fryns et le syndrome de Donnai-Barrow sont deux syndromes récessifs autosomiques dans lesquels une HDC est fréquemment présente (dans 80 et 70% des cas, respectivement). Pour le syndrome de Fryns, aucune mutation n'a encore été mise en évidence. Plusieurs syndromes monogéniques ont été décrits, les plus communs sont répertoriés dans le tableau 1.

Une même mutation dans le gène suppresseur de tumeur *Wt1* a été identifiée dans 3 cas de syndrome de Denys-Drash avec hernie diaphragmatique (Devriendt *et al*, 1995 ; Cho *et al*, 2006 ; Antonius *et al*, 2008). Il ne s'agit pas de la mutation la plus commune décrite dans ce syndrome, mais d'une mutation rare rapportée chez seulement 10 patients. Par contre les 7 autres patients porteurs de cette mutation rare ne présentent pas de HDC, ce qui suggère l'implication d'autres mécanismes dans la genèse des HDC. Il a été montré que des souris invalidées pour le gène *Wt1* développent en plus des malformations uro-génitales attendues, des hernies diaphragmatiques ce qui pourrait expliquer l'association fréquente de ces malformations chez l'homme (Kreidberg *et al*, 1993). Dans une étude récente, Slavotinek (2007) souligne l'intérêt qu'il y aurait à rechercher de façon systématique des mutations dans les gènes impliqués dans ces syndromes monogéniques en cas de HDC. Cependant, Nordenskjold *et al* (1996) ont analysé le gène *Wt1* chez 21 enfants porteurs de HDC isolées et chez 7 enfants avec malformations associées sans identifier de mutation.

Tableau 1: Syndromes monogéniques avec HDC

Syndrome	OMIM	Gène	Localisation
Simpson-Golabi-Behmel	312870	*GPC3* (glypican-3)	Xq26.1
Denys-Drash	194080	*WT1* (Wilms tumor 1)	11p13
Donnai-Barrow	222448	*LRP2* (low density lipoprotein-related protein 2)	2q31.1
spondylocostal dysostosis	277300	*DLL3* (delta-like-3)*	19q13.2
Matthew-Wood	601186	*STRA6* (stimulated by retinoic acid gene 6 homolog)	15q24.1
craniofrontal dysplasia	304110	*EFNB1* (Ephrin B1)	Xq12
Cornelia de Lange	122470	*NIPBL* (nipped-B-like)	5p13.1
Marfan	154700	*FBN1* (fibrillin 1)	15q21.1
Ehlers-Danlos type IV and type VII	130050 130060	*COL3A1* (collagen type III), *COL1A1* and *COL1A2*	2q31, 17q21-q22, 7q22.1

* gène le plus souvent muté

3) Les HDC associées à une anomalie chromosomique

Environ 15% des HDC sont associées à une anomalie chromosomique, la HDC étant alors un des éléments d'un syndrome polymalformatif. Les plus fréquentes sont la trisomie 21, la trisomie 13 et surtout la trisomie 18. Plus rarement, une tétrasomie 12p en mosaïque peut être retrouvée. Elle correspond au syndrome de Pallister Killian qui associe un hydramnios, une HDC, une croissance normale ou une macrosomie, une dysmorphie faciale, des fémurs courts, mais aussi une hypoplasie distale des doigts et des malformations cardiaques et urinaires. Enfin, des HDC ont été décrites dans le syndrome de Wolf Hirshhorn (Tachdjian *et al*, 1992 ; Van Dooren *et al*, 2004) et dans des cas de trisomie 22, notamment liées à la présence d'un marqueur surnuméraire correspondant à un der(22) issu d'une translocation 11;22 (Phillipson *et al*, 1990 ; Kim *et al*, 1992, Ladonne *et al*, 1996 ; Kadir *et al*, 1997).

Outre ces anomalies relativement fréquentes, de nombreuses autres anomalies déséquilibrées ont été décrites. Elles concernent une vingtaine de régions chromosomiques susceptibles de contenir des gènes dont un dosage génique anormal serait responsable de la HDC (Lurie, 2003).

Récemment, grâce aux techniques moléculaires de cytogénétique, notamment la CGH-array, des microdélétions ont été mises en évidence et certaines de façon récurrente, comme les microdélétions 8p23.1 (Lurie, 2003 ; Barber *et al*, 2005b ; Shimokawa *et al*, 2005 ; Faivre *et al*, 1998 ; Pecile *et al*, 1990), 15q24 et 15q26 (Slavotinek *et al*, 2005b ; Klaassens *et al*, 2005 ; Kantarci *et al*, 2006 ; Slavotinek *et al*, 2006 ; Scott *et al*, 2007b). La

mise en évidence de ces anomalies chromosomiques récurrentes a conduit à l'identification de gènes candidats comme NR2F2 (COUP-TFII) et MEF2A en 15q26.1-q26.2, ou GATA4 en 8p23.1 (Biggio *et al*, 2004 ; Klaassens *et al*, 2005). Le gène ST8SIA2 (MIM 602546) a également été proposé comme candidat dans la région 15q26 (Castiglia *et al*, 2005 ; Li *et al*, 2008). Ce gène est exprimé dans de nombreux tissus durant le développement et permettrait la polysialysation de molécules d'adhérence neuronales favorisant la migration cellulaire. Durant le développement du diaphragme il interviendrait à chaque stade de la myogenèse. Une mutation a été mise en évidence dans ce gène chez un patient porteur d'une HDC (Slavotinek *et al*, 2006). Nous reverrons en détail le rôle de certains de ces gènes dans le chapitre 3. Récemment, une étude a montré que la région 15q26 contenait un cluster de gènes exprimés dans le diaphragme en développement chez le rongeur, confortant la relation entre cette région et la HDC (Clugston *et al*, 2008). Les protéines correspondant aux gènes candidats pour la HDC localisés dans cette région chromosomique sont exprimées uniquement dans le diaphragme non musculaire, ceci confortant l'hypothèse d'une implication du tissu mésenchymateux dans la genèse des HDC. Enfin cette même étude montre que ces gènes candidats sont tous co-exprimés dans de mêmes cellules au sein du mésenchyme et donc qu'ils appartiennent probablement tous à une même voie de signalisation.

Une haploinsuffisance de la région 11p13, comprenant les gènes Wt1 et PAX6, a également été proposée comme facteur prédisposant au développement de HDC (Scott *et al*, 2005).

Certaines de ces anomalies chromosomiques (duplication 1q24-q31.2, délétion 1q41-q42, délétion 6q terminale, délétions 8p23.1 et 15q26) sont associées à un phénotype « Fryns-like » (Slavotinek *et al*, 2005b ; Clark et Fenner-Gonzales, 1989 ; Krassikoff et Sekhon, 1990 ; De Jong *et al*, 1989 ; Dean *et al*, 1991 ; Kantarci *et al*, 2006). Ceci suggère l'intérêt de rechercher de façon systématique la présence d'une anomalie chromosomique au caryotype standard, mais également de microdéséquilibres génomiques, notamment en cas de HDC syndromique.

Dans de rares cas, la HDC peut être associée à une translocation équilibrée (Howe *et al*, 1996 ; Enns *et al*, 1998). La recherche de gènes candidats peut alors se faire de façon très ciblée, au niveau des points de cassure. L'étude de ces translocations est à l'origine de la découverte de nombreux gènes impliqués dans divers syndromes (Bache *et al*, 2006).

Enfin, il est intéressant de remarquer que pour une même anomalie chromosomique, la présence d'une HDC est retrouvée de façon inconstante. Comme nous l'avons vu dans la deuxième partie de ce travail, d'autres facteurs génétiques ou

épigénétiques pourraient jouer un rôle dans la survenue de ces HDC dont l'origine est probablement multifactorielle.

4) Les HDC et la voie de signalisation des rétinoïdes

L'implication des rétinoïdes dans la genèse des hernies diaphragmatiques a été démontrée à plusieurs reprises. Cependant, les mécanismes moléculaires à l'origine de cette malformation restent encore mal connus.

a) La voie de signalisation de l'acide rétinoïque (Fig. 6)

L'acide rétinoïque (AR), principal dérivé biologiquement actif de la vitamine A ou rétinol, est un puissant modulateur de la croissance et de la différenciation de nombreux types cellulaires et joue un rôle central dans les processus de développement de l'embryon et dans l'homéostasie des tissus adultes.

Le rétinol est absorbé au niveau intestinal puis stocké au niveau hépatique (rétinylesters). Mobilisé, il est transporté lié à la RBP (*retinol-binding protein*) dans le plasma. Le complexe RBP-rétinol circule lié à une protéine – la transthyrétine – qui transporte également la thyroxine. Le complexe RBP-rétinol se fixe au niveau d'un récepteur membranaire, STRA6 (*stimulated by retinoic acid 6*), et seul le rétinol libre pénètre à l'intérieur de la cellule cible, où il se lie à un récepteur cytosolique, le CRBP (*cellular retinol-binding protein*).

Deux voies métaboliques sont possibles au sein de la cellule pour le rétinol :

- la voie de transformation en acide rétinoïque : le rétinol est dans un premier temps métabolisé en rétinaldéhyde sous l'influence d'une rétinol déshydrogénase (RDH) ou alcool deshydrogénase (ADH), puis en acide *tout-trans*-rétinoïque (ATTR) et en *9-cis* AR par une aldéhyde déshydrogénase (RALDH) ;
- la voie d'estérification : le rétinol non métabolisé en acide rétinoïque, est stocké sous forme d'esters non actifs. Ce stockage, hépatique essentiellement mais existant également au niveau du poumon et du placenta lors de la gestation, ainsi que la capacité cellulaire de mobiliser du rétinol assurent l'homéostasie du rétinol dans le plasma sanguin malgré les fluctuations dans la prise quotidienne alimentaire de vitamine A (Sapin *et al*, 2000).

L'ATTR se lie au sein du cytosol à une CRABP (cellular retinoic acid binding protein, cf. ci-dessous), puis pénètre à l'intérieur du noyau pour exercer son action après liaison avec des récepteurs nucléaires.

L'ATTR cellulaire est dégradé par des mono-oxygénases dépendant du cytochrome P450 (CYP26), en métabolites polaires moins actifs (acides 4-hydroxy-, 4-céto- et 4-oxo-rétinoïques).

b) les protéines de liaisons intracellulaires

Le rétinol entrant dans le cytoplasme est pris en charge par les CRBP (ou RBP) 1 et 2 (Cellular Retinol Binding Proteins) qui vont orienter le rétinol soit vers la voie de stockage par une enzyme, la LRAT (lécithine : rétinol acyltransférase) permettant la conversion du rétinol en rétinyl esters soit vers la synthèse en acide rétinoïque (Napoli, 1999).

Deux protéines cytoplasmiques lient l'AR dans la cellule : les CRABP 1 et 2, avec une affinité différente en fonction des isoformes de l'AR. L'affinité de CRABP1 pour l'AR est supérieure à celle de CRABP2. Les CRABP jouent un rôle dans le transport de l'ATTR vers son site d'action nucléaire. CRABP1 a une plus large distribution tissulaire. CRABP2 est surtout exprimée durant l'embryogenèse et dans les kératinocytes. Les CRABP moduleraient ainsi la voie de signalisation de l'AR en le séquestrant ou en intervenant sur son catabolisme par son transport vers le réticulum endoplasmique et le cytochrome P450 associé, permettant de contrôler la concentration d'AR libre et donc l'activation des récepteurs nucléaires (Napoli, 1993, 1996 et 1999). CRABP2 aurait également une localisation nucléaire et un rôle dans la transactivation des gènes sensibles à l'AR en participant au complexe transcriptionnel des récepteurs de l'AR (Delva *et al*, 1999).

Le transfert de l'AR de CRABP2 vers les récepteurs nucléaires implique une interaction directe entre CRABP2 et le récepteur nucléaire. Les CRABPs pourraient également constituer des mécanismes de protection de la cellule contre des concentrations trop élevées et toxiques de formes libres d'ATTR.

c) Les enzymes du métabolisme cellulaire du rétinol

- conversion du rétinol en rétinal :

Les alcool deshydrogénases (ADH) appartiennent à la famille des deshydrogénases / réductases à chaîne moyenne (Jörnvall *et al*, 1995a ; Duester *et al*, 1999). Six classes existent chez les mammifères et les gènes correspondant sont localisés dans une même région chromosomique (4q22-q23 chez l'homme), organisés en clusters dans le même ordre et avec la même orientation transcriptionnelle (Edenberg, 2000 ; Szalai *et al*, 2002). Cette extrême conservation de la structure des gènes suggère que ces ADH ont

des fonctions très conservées chez les mammifères. L'ADH3 est ubiquitaire alors que les autres ADH ont une expression variable en fonction des tissus (Edenberg, 2000). D'autres enzymes appartenant à la famille des deshydrogénases / réductases à chaîne courte (SDR) sont capables de convertir le rétinol en rétinal : les RODH-like (rétinol deshydrogénases) (Jörnvall *et al*, 1995b). Ce sont des enzymes microsomales. Elles sont souvent coexprimées avec CRBP1 qui agirait comme une protéine chaperonne pour le rétinol et le rétinal, les dirigeant vers les enzymes du métabolisme (Everts *et al*, 2005).
Enfin, plus récemment, les aldo-kéto réductases (AKR) ont été définies comme un nouveau groupe d'enzymes cytosoliques pouvant contribuer aux conversions oxydoréductives des rétinoïdes (Crosas *et al*, 2003). Deux d'entre elles, AKR1B1 et AKR1B10, auraient une activité rétinaldéhyde réductase chez l'homme (Gallego *et al*, 2006).

- conversion du rétinal en acide rétinoïque :
Les aldéhydes deshydrogénases (RALDH) sont capables de catalyser irréversiblement et spécifiquement l'étape finale de synthèse de l'AR. Il existe trois RALDH distinctes : RALDH1, RALDH2 et RALDH3 (Perozich *et al*, 1999). RALDH2 est très conservée lors de l'évolution et de nombreuses études chez la souris ont montré qu'elle était indispensable pour le développement embryonnaire précoce (Niederreither *et al*, 1999, 2000 et 2001). Lors du développement fœtal chez la souris, les 3 gènes sont exprimés de façon variable en fonction des organes ce qui montre qu'il existe une régulation fine du niveau d'expression de ces gènes lors de l'organogenèse (Niederreither *et al*, 2002). RALDH1 est essentiellement exprimé dans le poumon en développement (épithélium bronchique et trachéal) et RALDH2 est exprimé principalement dans les tissus mésenchymateux. Il semblerait que RALDH1 ne soit pas essentielle pour la synthèse de l'AR dans de nombreux tissus (Duester *et al*, 2003). Récemment il a été montré que RALDH1 serait impliquée dans le catabolisme du rétinol en excès (Blomhoff et Blomhoff, 2006). Une RALDH4 a été identifiée chez la souris avec un profil d'expression restreint (foie fœtal et rein adulte) et une plus grande affinité pour le 9-cis rétinal que pour le rétinal tout-trans (Lin *et al*, 2003).

- dégradation de l'AR :
Le catabolisme de l'AR est essentiel puisqu'il participe à la régulation de la concentration d'AR dans la cellule et donc indirectement à la modulation de l'expression de certains gènes. Les cytochromes spécifiquement impliqués dans le catabolisme de l'AR sont des hydroxylases appartenant à la sous-famille 26 des cytochromes P450 (CYP26). On

distingue trois gènes : CYP26A1, B1 et C1, dont les profils d'expression sont variables, ce qui suggère des rôles différents pour chacune de ces enzymes (Reijntjes *et al*, 2004). La région proximale en amont du promoteur du gène de CYPA1 contient un RARE fonctionnel ; sa transcription est donc inductible par l'AR (Loudig *et al*, 2000). CYP26C1 a une affinité pour le 9-cis AR bien supérieure à celle de CYP26A1 et B1. Plusieurs produits issus de la dégradation comme l'acide 4-oxo rétinoïque, l'acide 18-hydroxy rétinoïque et le rétinoyl β-glucuronide, restent biologiquement actifs (Reijntjes *et al*, 2005).

d) les récepteurs nucléaires RAR et RXR

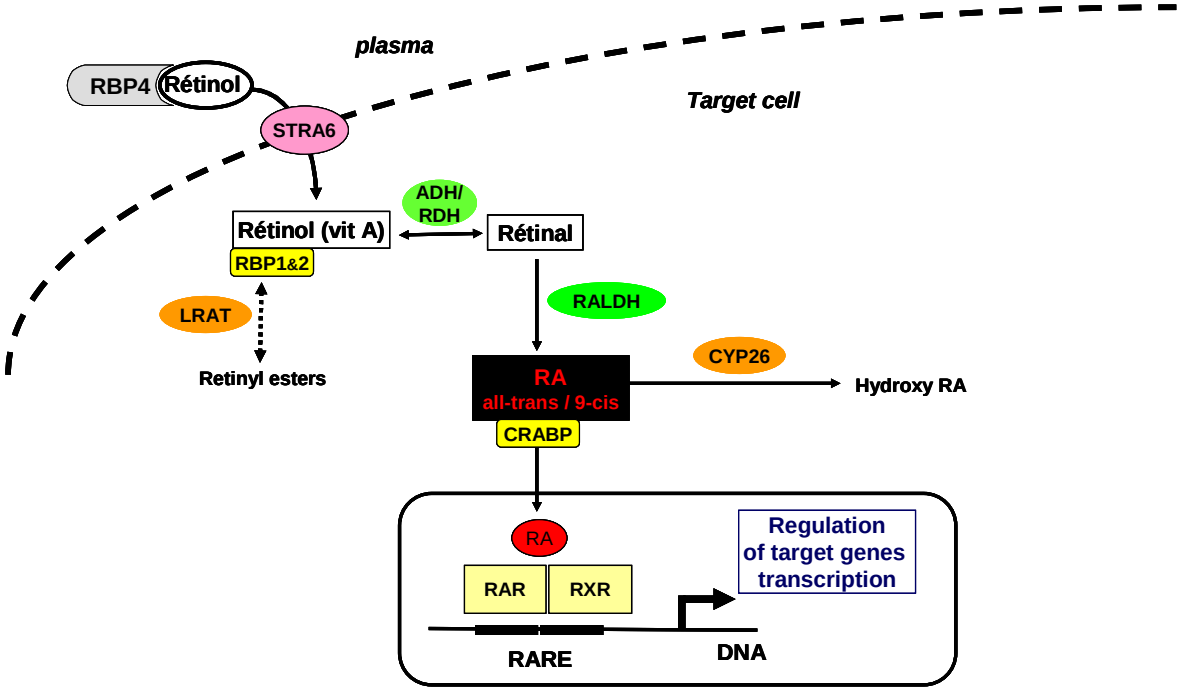
Les récepteurs nucléaires aux rétinoïdes appartiennent à la superfamille des récepteurs nucléaires aux stéroïdes, aux hormones thyroïdiennes et à la vitamine D3. Les deux familles de récepteurs RAR (Retinoic Acid Receptors) et RXR (Retinoic X Receptors) possèdent chacune trois isotypes α, β et γ codés par des gènes différents permettant ainsi de nombreuses combinaisons de cette unité fonctionnelle, probablement à l'origine de la diversité des effets biologiques de l'AR dans l'organisme.

L'action pharmacologique de l'AR passe par sa liaison à ces récepteurs nucléaires. L'AR se lie à ces récepteurs intra-nucléaires RAR et RXR qui, après hétérodimérisation, interagissent avec l'ADN au niveau d'éléments de réponse spécifiques ; les RARE (Retinoic Acid Responsive Element) modulant la transcription de nombreux gènes cibles (Balmer et Blomhoff, 2002). RAR est capable de fixer l'ARTT et le 9-cis AR alors que RXR fixe préférentiellement le 9-cis.

La liaison du ligand au récepteur entraîne une modulation transcriptionnelle de gènes cibles (codant des facteurs de croissance, des oncogènes, des kératines, de l'élastine et diverses d'enzymes comme la transglutaminase), base de l'action pharmacologique de ces molécules.

Les récepteurs dérivés du gène codant RARα sont mis en évidence dans de nombreux tissus embryonnaires et adultes et seraient responsables des effets des rétinoïdes sur la croissance et la différenciation. Une revue récente de la littérature a fait une synthèse de l'expression de ces récepteurs à différents stades du développement et dans différents organes afin de mieux comprendre leur rôle respectif (Dollé, 2009). Cette étude montre que RARα, RXRα et RXRβ ont une expression ubiquitaire alors que RARα et RARγ ne sont exprimés que dans certains organes ou types cellulaires et que le profil d'expression de RXRγ est encore plus restreint.

FIGURE 6 : Représentation schématique de la voie de signalisation des rétinoïdes



e) Rôle des rétinoïdes dans la HDC

De nombreuses études ont montré que les rétinoïdes jouent un rôle important dans le développement du diaphragme (Kluth *et al*, 1996 ; Greer *et al*, 2000 ; Gallot *et al*, 2005). La majorité des liens évoqués entre la HDC et les rétinoïdes a été établie à partir de modèles murins.

Chez la souris, la déplétion en vitamine A durant la gestation entraîne l'apparition d'une hernie diaphragmatique chez un quart des fœtus (Anderson, 1941 et 1949). Le nombre de fœtus atteints diminue lorsque la vitamine A est réintroduite dans l'alimentation des rates en milieu de gestation (Wilson *et al*, 1953). Durant la gestation, une exposition au nitrofène, un herbicide inhibant la synthèse d'acide rétinoïque par inhibition de RALDH2, est également à l'origine de l'apparition de HDC chez 80% des fœtus et l'administration de vitamine A permet de rétablir le phénotype (Chen M *et al*, 2003, Mey *et al*, 2003, Babiuk *et al*, 2004 ; Thebaud *et al*, 1999). Enfin, la double invalidation RARβ/RARα chez la souris est à l'origine de nombreuses malformations dont des hernies diaphragmatiques (Mendelsohn *et al*, 1994).

Chez l'homme, une seule étude clinique de faible effectif a rapporté une diminution de 50% du taux plasmatique en rétinol et RBP dans le sang du cordon de nouveaux nés porteurs de hernies diaphragmatiques (Major *et al*, 1998). Récemment, la mise en évidence de mutations dans le gène *STRA6*, récepteur membranaire du rétinol, chez des patients porteurs d'un syndrome polymalformatif incluant une HDC vient renforcer cette hypothèse « rétinoïdes » (Pasutto *et al*, 2007 ; Golzio *et al*, 2007). Il s'agit de la première altération phénotypique associée à une mutation d'un gène appartenant à la voie des rétinoïdes décrite chez l'homme.

III/ Recherche de gènes candidats rétinoïdes-dépendants dans les régions chromosomiques remaniées de façon récurrente en cas de HDC

Pour renforcer l'hypothèse « rétinoïdes », nous avons recherché, à l'aide de différentes bases de données comme OMIM ou UCSC, la présence de gènes intervenant dans la voie de signalisation des rétinoïdes ou régulés par l'AR et situés dans des régions chromosomiques remaniées de façon récurrente chez des patients porteurs de HDC isolée ou non. Ce travail fait l'objet d'un article sous forme de revue de la littérature (Publication n°9, acceptée le 16/10/2009 dans Fetal Diagnosis and Therapy).

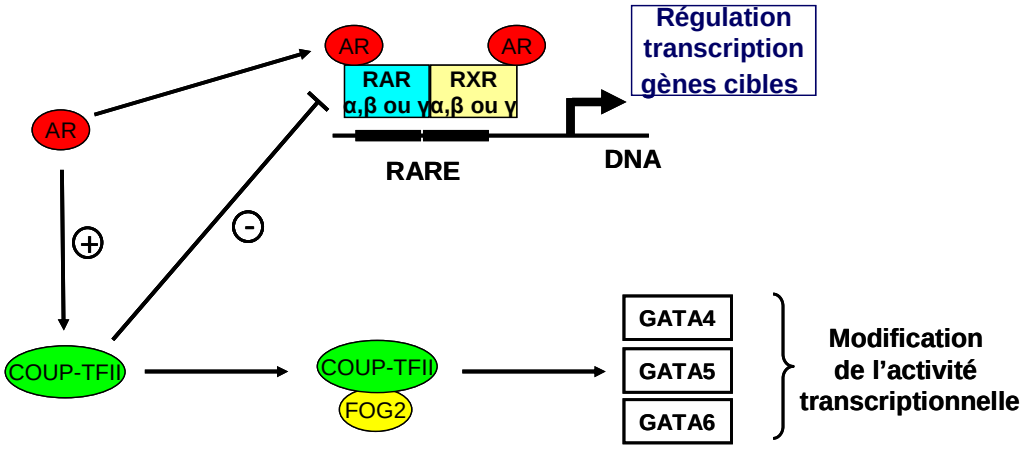
Nous proposons 13 gènes candidats dont certains avaient déjà été décrits comme les gènes GATA4, FOG2 et COUP-TFII, localisées respectivement en 8p23.1, 8q23.1 et 15q26.1-q26.2, régions chromosomique fréquemment délétées chez des patients porteurs de HDC. Ces 3 gènes interviennent dans la voie de signalisation des rétinoïdes et jouent un rôle clé dans le développement du diaphragme (Ackerman *et al*, 2005 ; You *et al*, 2005 ; Jay *et al*, 2007). COUP-TFII et FOG2 interagissent et sont capables de modifier l'expression de nombreux gènes dont celle de *GATA4* (Holder *et al*, 2007). COUP-TFII est également capable d'inhiber l'effet de l'AR sur les récepteurs nucléaires en séquestrant RXR, empêchant ainsi leur hétérodimérisation (Fig. 7). Par ailleurs, il a été montré récemment que ces 3 gènes étaient co-exprimés au niveau des cellules mésenchymateuses des membranes pleuropéritonéales (Clugston *et al*, 2008). Nous proposons d'autres gènes candidats comme STRA6, LRAT, CRBP1, CRBP2 et CRABP1 qui sont directement impliqués dans le métabolisme de l'AR.

L'intérêt d'identifier ces gènes susceptibles de jouer un rôle dans le développement du diaphragme est essentiellement diagnostic. En effet, des mutations ont été récemment

mises en évidence dans le gène *FOG2* chez des patients présentant des HDC isolées (Ackerman *et al*, 2005 ; Bleyl *et al*, 2007).

Dans l'avenir il serait intéressant de rechercher des déséquilibres ou des mutations de ces gènes candidats pouvant être à l'origine de ces HDC.

FIGURE 7 : Représentation schématique des mécanismes de régulation de la voie des rétinoïdes
d'après Holder et al, 2007



D'après Holder et al, AM J Hum genet 2007

L'AR augmente l'expression de COUP-TFII qui va : 1) interagir avec FOG2 capable de moduler la transcription de GAT4, GATA5 et GATA6 ; 2) par séquestration de RXR, empêcher l'hétérodimérisation de RAR et RXR et donc effectuer un rétrocontrole négatif

IV/ Etude de l'expression des acteurs de la voie de signalisation des rétinoïdes dans des fibroblastes de fœtus porteurs d'une HDC

Comme nous l'avons dit précédemment, très peu d'études chez l'homme montrent le lien entre rétinoïdes et HDC. Cette malformation est rare et il est souvent difficile d'obtenir de l'ARNm de bonne qualité en quantité suffisante. De plus, il est difficile d'induire une prolifération cellulaire en culture à partir de prélèvements pulmonaires ou diaphragmatiques. Afin de remédier à ces problèmes, nous proposons comme modèle

cellulaire les fibroblastes cutanés fœtaux pour étudier la voie de signalisation des rétinoïdes chez des fœtus porteurs de HDC. En effet, ces fibroblastes cutanés ont la même origine mésoblastique que le contingent mésenchymateux des membranes pleuropéritonéales dont l'absence ou le défaut de développement serait à l'origine des HDC (Clugston, 2007).

Pour valider ce modèle, nous avons étudié l'expression de différents acteurs impliqués dans la voie de signalisation des rétinoïdes dans des fibroblastes cutanés fœtaux en culture primaire.

La présence des transcrits codants pour les acteurs du signal rétinoïque [récepteurs nucléaires (RARs et RXRs), enzymes du métabolisme (ADHs, RALDHs, CYP26s) et protéines de liaison (CRBPs, CRABPs)] a été recherchée par RT-PCR.
Pour les récepteurs nucléaires nous avons montré que les récepteurs RARα, β et γ et RXRα sont exprimés dans les fibroblastes cutanés fœtaux alors que les RXR β et γ ne le sont pas. Les profils protéiques étudiés par immunocytologie en fluorescence confirment ces résultats.

En ce qui concerne les enzymes du métabolisme des rétinoïdes et les protéines de liaison, au moins une isoforme de chacune des protéines est exprimée. Ceci signifie que tous les acteurs du signal sont représentés dans les fibroblastes fœtaux pour rendre cette voie de signalisation fonctionnelle. Il semblerait donc que les fibroblastes cutanés fœtaux soient un bon modèle d'étude *in vitro* de la voie de signalisation des rétinoïdes chez l'homme.

Ayant validé ce modèle, nous avons ensuite réalisé ce même profil d'expression dans des fibroblastes cutanés de fœtus porteurs de HDC isolées ou associées à d'autres malformations et/ou anomalies chromosomiques. La majorité des fœtus avec HDC présentaient le même profil d'expression que les fœtus témoins.

Chez un fœtus porteur d'une tétrasomie 12p homogène au caryotype après culture cellulaire, aucun des ARNm des trois RAR n'a été détecté. Nous pouvons suggérer que l'absence des trois RAR est à l'origine de la HDC chez ce fœtus puisque chez la souris la double invalidation pour RARα et β ou le blocage de RAR par un antagoniste sont à l'origine de HDC (Mendelsohn *et al*, 1994 ; Clugston *et al*, 2009). Chez ce même fœtus, l'ARNm d'aucune des trois enzymes de dégradation (CYP26) n'a été détecté. Comme nous l'avons dit précédemment, le catabolisme de l'AR est essentiel puisqu'il permet de réguler les concentrations d'AR dans la cellule et donc indirectement l'expression de certains gènes.

Chez un fœtus porteur d'une HDC gauche isolée, l'ARNm de RALDH2 est indétectable. Cette enzyme, exprimée principalement dans les tissus mésenchymateux, joue

un rôle clé dans le métabolisme des rétinoïdes. De plus, comme nous l'avons vu précédemment, des HDC sont présentes chez les fœtus de rates exposées au nitrofène, herbicide inhibant l'activité enzymatique de RALDH2 (Thebaud *et al*, 1999 ; Chen M *et al*, 2003 ; Mey *et al*, 2003 ; Babiuk *et al*, 2004). Enfin, Clugston *et al* (2009) ont montré récemment que RALDH2 était responsable de la synthèse d'AR au niveau des membranes pleuropéritonéales. Nous pensons donc que l'absence d'expression de RALDH2 pourrait être à l'origine de la HDC chez ce fœtus.

Ces données préliminaires suggèrent que dans l'avenir il pourrait être intéressant de rechercher des mutations ou des déséquilibres dans ces gènes (RAR, CYP26 et RALDH2) chez des patients porteurs de HDC afin d'établir leur réelle implication dans la genèse des HDC.

Ce travail a fait l'objet d'un article accepté dans Birth Defect Research part A (publication n°10).

V/ Perspectives

1) Recherche de déséquilibres génomiques chez des fœtus avec HDC

A ce jour, nos travaux portent sur 21 fœtus ou nouveaux nés porteurs de HDC isolée ou associée à d'autres malformations (Tableau 2). Les examens cytogénétiques ont été réalisés à partir de prélèvements de liquide amniotique ou de biopsies cutanés pratiquées lors de l'autopsie ou lors de la chirurgie réparatrice. Un caryotype standard a été systématiquement réalisé ainsi qu'une AneuVysion et une FISH en direct à la recherche d'une tétrasomie 12p (sonde CEP12) pour les cas diagnostiqués en prénatal.

Depuis janvier 2004, dans le cadre d'un protocole de recherche clinique, chez les fœtus avec HDC et sans anomalie au caryotype, des investigations complémentaires sont réalisées afin de rechercher des déséquilibres génomiques cryptiques (FISH, CGH sur métaphase, MLPA avec kits P036D, P070 et P245).

patients	terme (SA)	prélèvements	côté	malformations associées	caryotype	issue grossesse
1	23	LA - fibroblastes	G	-	46,XY	IMG
2	15	Fibroblastes	G	Hygroma kystique cervical > 6mm	46,XX	IMG
3	25	LA - fibroblastes	G	Dysmorphie faciale	47,XY,i(12p)	IMG
4	23	LA - fibroblastes	G	CIV, dysmorphie, pieds bots	46,XX,+18	IMG
5	40	LA - fibroblastes	G	-	46,XX	Né, va bien
6	35	Fibroblastes	D	-	46,XY	IMG
7	Naissance	Sang - fibroblastes	D	Dysmorphie faciale	46,XX	Décès néonatal
8	24	LA - fibroblastes	G	-	46,XY,inv(1)(p11q13)	Décès néonatal
9	24	LA - fibroblastes	centrale	Fente labio-palatine, pied bot, CIA	46,XY	IMG
10	33	LA	G	-	46,XX	Né, va bien
11	30	LA - fibroblastes	D	CIA	46,XX	IMG
12	14	LA - fibroblastes	G	CN élevée	46,XY	IMG
13	33	LA	G	Agénésie corps calleux, hypogénitalisme, hypotrophie, dysmorphie faciale	47,XY,+der(22)t(11;22)mat	Né
14	31	LA - fibroblastes	G	Hypoplasie globes oculaires, dysmorphie, duplication utérine Mathew Wood ?	46,XX	IMG, pas de mutation de STRA6
15	27	LA	G	-	46,XY	Né, va bien
16	30	LA	G	-	46,XX	Né, va bien
17	26	LA	G	-	46,XY	Né, va bien
18	25	LA	G	Reins hyperéchogènes	46,XY	Né, mutation TCF2 (MODY5)
19	24	LA	G	-	46,XY	Né, va bien
20	24	LA - fibroblastes	G	-	46,XX	IMG
21	25	LA		Dysmorphie, RCIU	46,XY,+18	IMG

Tableau 2 : Fœtus/nouveaux nés porteurs d'une HDC

Ainsi la recherche de microdélétions 8p23.1, 15q26.2 et 15q26.3 a été faite par FISH avec des sondes « à façon » fabriquées à partir de BAC (Chromosomes Artificiels de Bactéries) ciblant respectivement les gènes candidats GATA4, COUP-TF2 et MEF2A [Myocyte Enhancer Factor 2A] (Fig. 8) (protocole en annexe). Le gène MEF2A, proposé comme gène candidat par Biggio *et al* en 2004, joue un rôle important dans le contrôle de la différentiation et le développement musculaire.

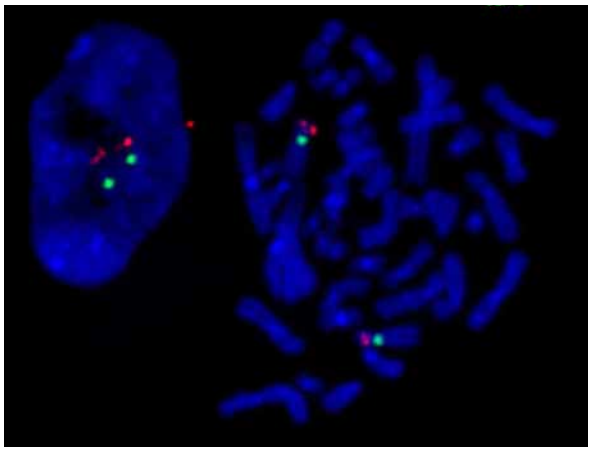
Pour les techniques de CGH (protocole décrit dans la publication n°5) et de MLPA (protocole en annexe), les extractions d'ADN à partir des culots cellulaires ont été faites au DNAzol [Invitrogen] de 2004 à 2007 et à partir de 2008 à l'aide du kit NucleoSpin Blood [Macherey Nagel]. Enfin, ces prélèvements ont tous été cryoconservés afin de pouvoir réaliser des explorations complémentaires.

Quatre anomalies chromosomiques déséquilibrées ont été mises en évidence par FISH et/ou au caryotype standard (Tableau 2) : deux trisomies 18, une tétrasomie 12p correspondant au syndrome de Pallister Killian et un dérivé 22 d'une translocation 11;22. Ce dernier cas a fait l'objet d'une publication (Gremeau *et al*, 2009, publication N°11).

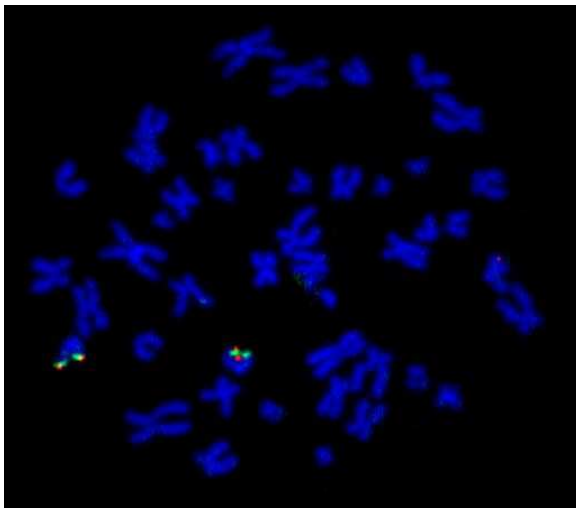
A ce jour, aucune microdélétion 8p23.1 ni 15q26 n'a été mise en évidence par FISH. La CGH sur métaphase et la MLPA n'ont pas mis en évidence de déséquilibre génomique.

Nous souhaitons poursuivre ces recherches de déséquilibre génomique avec une CGH array pangénomique haute résolution (puce 180 K avec une distance moyenne entre deux clones de 17Kb). Les éventuels déséquilibres ainsi mis en évidence de façon significative nous permettrons d'identifier de nouveaux gènes candidats et peut être également des polymorphismes de nombre de copies de séquence (CNVs) pouvant être à l'origine d'une prédisposition à développer une HDC.

FIGURE 8 : FISH à façon « gènes candidats »



a) CEP8 / GATA4 (RP11-235I5 en 8p23.1)



b) COUP-TFII (RP11-337N12 en 15q26.1) /
MEF2A (RP11-530L17 en 15q26.3)

2) Etude des gènes candidats

Nous avons identifié de nombreux gènes candidats impliqués dans le métabolisme des rétinoïdes. Nous envisageons d'étudier ces gènes par QMPSF chez les fœtus porteurs d'une HDC à caryotype normal afin de mettre en évidence d'éventuels déséquilibres pouvant confirmer leur implication dans la genèse des HDC.
Dans un second temps nous souhaitons étudier l'expression de ces gènes dans les fibroblastes des fœtus porteurs d'une HDC grâce à la technologie GeXP (Beckman Coulter) pour l'analyse multiplex de l'expression de gène.

VI/ Conclusion

La détection prénatale de la HDC est une situation de plus en plus fréquente. A ce jour, seuls des examens de cytogénétique sont réalisés de façon systématique afin d'éliminer une anomalie chromosomique.

Dans l'avenir, l'établissement de corrélations génotype-phénotype fines pourrait permettre de développer des stratégies diagnostiques adaptées à chaque patient et d'envisager un diagnostic prénatal moléculaire ciblé en cas de découverte d'une HDC *in utero*.

Publications n° 9 à 11

Retinoid pathway and congenital diaphragmatic hernia:

hypothesis from the analysis of chromosomal abnormalities

Accepté dans Fetal Diagnosis and Therapy

Running Title: Retinoid and CDH chromosomal loci

GOUMY Carole[1], GOUAS Laetitia[1], MARCEAU Geoffroy[2], COSTE Karen[2], VERONESE Lauren[1], GALLOT Denis[1], SAPIN Vincent[2], VAGO Philippe[1], TCHIRKOV Andrei[1]

[1]Univ Clermont 1, UFR Médecine, Histologie Embryologie Cytogénétique, Clermont-Ferrand, F-63001 France; CHU Clermont-Ferrand, Cytogénétique Médicale, Clermont-Ferrand, F-63003 France.

[2] GReD, CNRS UMR6247 – Clermont Université - INSERM U931, UFR Médecine, Clermont-Ferrand, F-63011 France.

Correspondance to:

Docteur Carole GOUMY

Cytogénétique Médicale

Faculté de Médecine – BP 38

63001 Clermont-Ferrand Cedex – France

Tel : +33 (0)4 73 17 81 00

Fax : +33 (0)4 73 26 91 82

E-mail: cgoumy@chu-clermontferrand.fr

ABSTRACT (269 words)

Background/Objectives: Although there is strong evidence implicating genetic factors in CDH pathogenesis, few causal genes have been identified. Many studies suggest that early disruption of the retinoid signaling pathway during gestation may contribute to CDH etiology. Chromosome abnormalities are detected in 10-20% of CDH cases. Chromosomal regions that are involved in balanced translocations or are recurrently deleted or duplicated in patients with CDH are of particular interest to researchers, because they are more likely to harbor genes that cause or predispose to the development of CDH.

The aim of this review was to select chromosome loci which have been shown to be associated with CDH and to investigate if these loci contain candidate genes involved in retinoic signaling pathway.

Data Sources: We have reexamine the known CDH-critical chromosomal loci and search in available databases such as UCSC Genome Browser and OMIM whether candidate genes related to the retinoid pathway were present within these loci.

Results: Twelve retinoid related genes have been proposed as potential candidates. Among them, COUP-TFII, FOG2 and GATA4 have already been well studied in particular in animal models. We propose other candidates as STRA6, LRAT, CRBP1, CRBP2 and CRABP1 directly implicated in retinoic acid metabolism.

Conclusion: The identification of CDH-related genes and pathways affecting normal diaphragm will contribute to the understanding of the pathophysiology of this severe embryopathy and might help to facilitate prenatal management and devise more individual treatment strategies. Further studies are necessary to screen large cohorts of patients with CDH for microimbalances or *de novo* mutations in these candidate genes. Moreover, functional analyses are needed to establish their exact role in CDH etiology.

BACKGROUND/OBJECTIVES

CDH is a severe birth defect with an estimated prevalence of 1/3000 [1-4]. Posterolateral defects, named Bochdalek hernias, account for approximately 95% of CDH, with more than 80% of cases being left-sided. The defect in diaphragm development leads to the herniation of abdominal viscera into the chest cavity during the early stages of lung development. Newborns with CDH often have severe respiratory distress resulting from pulmonary hypoplasia. CDH occurs as an isolated birth defect (isolated CDH) or is associated with additional malformations (non-isolated CDH), such as cardiovascular defects, abnormalities of the CNS and urogenital anomalies. The development of the human diaphragm occurs between the 4^{th} and 12^{th} week of gestation. The primordial diaphragm development arises from four different structures: septum transversum, pleuroperitoneal folds, dorsal mesentery, and elements from the thoracic body wall [5,6]. Several theories have been proposed to explain primary embryologic events leading to CDH such as failure of closure of the pleuroperitoneal canals, defective myoblast formation or abnormal phrenic nerve innervation [7-9]. In animal models, CDH arises from a malformation of the amuscular mesenchymal substratum of the pleuroperitonel folds before pleuroperitoneal canal closure [10,11].

Some individuals with non-isolated CDH have patterns of anomalies that are strongly suggestive of a specific genetic syndrome (see Table 1) [12-17]. The same rare mutation in *WT1* has been reported in three cases of CDH with clinical features of Denys-Drash syndrome [18-20]. This gene encodes a zinc finger transcription factor expressed in the septum transversum and in the pleural and abdominal mesothelial tissues that form the diaphragm. Homozygous null mouse embryos for *WT1* develop diaphragmatic hernia [21]. Thus, WT1 appeared as a good candidate for CDH in humans even if in a screening study of 27 children any mutation was found [22]. Interestingly, this gene is located on chromosome 11p13, a region recurrently deleted in individuals with CDH [23]. Chromosomal abnormalities were identified in approximately 10-20% of CDH cases, the rate being higher in cases with associated malformations [24-28]. The existence of several chromosome “hot spots” suggests the presence of genes that cause or predispose to the development of CDH in these regions. Trisomies 18, more rarely trisomy 13 and 21, and structural chromosome abnormalities, such as the presence of a supernumerary derivative chromosome 22, have been described in association with CDH. CDH is also frequently present in the Pallister-Killian syndrome associated with a tetrasomy 12p [29-31]. Other chromosomal defects involving almost all the chromosome pairs have been described [25,32]. In the majority of published cases, chromosome abnormalities were

identified using R or G-banded analysis and FISH. Recently, high resolution techniques such as array-based Comparative Genomic Hybridization (aCGH) have revealed various small recurrent chromosome abnormalities in CDH patients and allowed a more precise breakpoint characterization facilitating the identification of CDH-related genes [33-38]. Several studies have suggested that 15q24-26 and 8p23.1 are critical for normal development of the diaphragm since recurrent deletions within these regions were associated with CDH [33-35,39-42]. CDH has also been reported in several cases of monosomy 4p16pter associated with Wolf-Hirshhorn syndrome [43-45]. Other candidate regions such as 1q41-q42, 6p22-p25, or 22q11 have also been described [23,36,38]. Balanced reciprocal translocations were also described in CDH patients [23,25]. These translocations might cause CDH by disrupting or inactivating specific genes, and the characterization of breakpoints in such cases may be a valuable approach to identify candidate genes [46].

The diaphragm development strongly depends on the role of proteins associated with the metabolism and binding of retinoids [47,48]. The nitrofen rat model has particularly highlighted the importance of retinoic acid (RA) in the diaphragm development, but this model has also provided limited insights into understanding the genetic basis of CDH [9]. Retinoids play a central role in many biological processes, particularly during embryogenesis and lung development [49-53]. The RA signaling pathway is complex but recent studies in several species have increased our understanding of the role of RA as a signaling molecule during vertebrate development [54,55]. Figure 1 represents a schematic overview of the RA signaling pathway. Numerous studies have revealed the role of a retinoid signaling pathway disruption in the pathogenesis of CDH [47,48,56]. In rodents, the first evidence linking retinoids with CDH comes from the observation that 25 to 40% of the offspring of rat dams that were fed a diet deficient in vitamin A developed CDH [57,58]. The number of affected pups decreased when vitamin A was reintroduced into the diet in mid-gestation [59]. A proportion of RAR double mutants in mice lacking both α and β subtypes exhibited a posterolateral diaphragmatic defect which is similar to that seen in humans [60]. *In utero* exposure to the nitrofen herbicide, that inhibits the enzymatic activity of RALDH2, a key molecule responsible for the conversion of retinal in RA, was shown to cause CDH and primary lung defects [61]. Recently, Clugston et al have shown that a blockage of RAR signaling with the pan-RAR antagonist BMS493 induced a very high degree of CDH with a marked left-right sideness that depended on the timing of drug delivery [62]. In humans, preliminary evidence that retinoids may play a role in CDH development comes from a small study in which retinol and retinol-binding-protein plasma levels were found to be

decreased by around 50% in newborns with CDH compared with healthy newborns [63]. Recently, the retinoid hypothesis has been reinforced by the identification of mutations in *STRA6* (stimulated by retinoic acid 6), a membrane receptor for retinol binding protein that mediates cellular uptake of vitamin A, in a pleiotropic malformation syndrome including CDH [15].

The aim of this review was to select chromosome loci which have been shown to be associated with CDH and to investigate if these loci contain candidate genes involved in retinoic signaling pathway. This overview of chromosomal hot spots and associated candidate genes could have future diagnostic and therapeutic interests in term of clinical management of congenital diaphragmatic hernia.

DATA SOURCES

This work was performed thanks to the collaboration between a cytogenetic laboratory and a research team working on the developmental implications of the active derivatives of retinoids for mammalian species since a few years.

We have first identified chromosomal loci recurrently affected in CDH context using an extensive review of the literature with the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed). Both Lurie and Enns et al. have published useful reviews of chromosomal anomalies associated with CDH [25,32]. Using these reviews as a foundation, we have compiled an updated list of the CDH-associated chromosomal anomalies. Then we have search in available databases such as UCSC Genome Browser (http://genome.ucsc.edu) and OMIM (http://www.ncbi.nlm.nih.gov/omim) whether candidate genes related to the retinoid pathway were present within the selected CDH-critical chromosomal loci.

More than twenty RA metabolic pathway genes are currently known. These genes are involved in vitamin A binding and transport (*RBP4, transthyretin, STRA6, CRBP1-2, CRABP1-2)*, storage (*LRAT, diacylglycerol acyltransferase*), intracellular RA synthesis (*ADH3-4, dehydrogenase/reductase (SDR family) member 4 and 9, retinol dehydrogenase 4, epimerase, RALDH1-4, aldo-keto reductase family 1, member B1 and B10)* and RA degradation (*CYP26A1, B1 and C1).* In addition, six genes encoding nuclear receptors are involved in the RA signal transduction (*3 RARs and 3 RXRs)* and many other genes such as *GATA4, FOG2* and *COUP-TFII* act as regulators of this pathway. Among all these genes, we identified 12 candidates in the selected chromosome regions.

RESULTS: CDH associated chromosomal hot spots and candidate genes involved in the retinoic acid pathway or retinoic acid regulated

Chromosome 1

1q41 : *DISP1* [MIM 607502]

There is emerging evidence that loss of one or more genes in the 1q41-q42 region predisposes to CDH [36,41,64-67]. Combining cytogenetic results from all published CDH cases, the smallest region of overlap is approximately 1.2 Mb. The *DISP1* (dispatched 1) gene could be the prime candidate gene in this region, through its interaction with Sonic hedgehog (Shh), a crucial protein for the patterning of the early respiratory system in the mouse embryo [67-69].

In the nitrofen rat model of CDH and in the hypoplasic lung of human fetuses with CDH it has been shown that Shh was downregulated [70]. It has been shown that COUP-TFII, a repressor of the retinoid pathway (Figure 1), is a target gene of Shh [71]. Then, it is tempting to speculate that a deregulation of the Shh pathway, through an alteration of DISP1, might disrupt the RA pathway by deregulating COUP-TFII and lead to CDH.

Recently, *HLX* [MIM 142995] has been proposed as a new candidate gene in this region because sequence variants have been identified in patients with isolated CDH [72].

Chromosome 3

3q23: *CRBP1 / RBP1* [MIM 180260] and *CRBP2 / RBP2* [MIM 180280]

Wolstenholme *et al.* have reported an association of blepharophimosis sequence and CDH in a child with a del(3)(q21q23) [73]. Dillon *et al* also reported CDH and del(3)(q22) in two patients [74]. Lurie suggested that 3q22 may harbor a gene that when deleted could lead to CDH [32]. *RBP1* (Retinol Binding Protein 1) and *RBP2* (Retinol Binding Protein 2) map at the distal end of the 3q23 band flanking 3q22. *RBP1* and *RBP2* encode cellular RBPs (CRBPs) involved in the intracellular movement of retinol [75] (Figure1).

These genes are part of the retinol signaling pathway and have been shown to play a role in vitamin A homeostasis and lung maturation in mice [10,76].

Chromosome 4

4q32.1: *LRAT* [MIM 604863]

CDH has been described in four individuals with 4q31 deletion and four individuals with duplication of this region [23]. The *LRAT* gene maps to 4q32.1 flanking 4q31.

The protein encoded by this gene is a microsomal enzyme that catalyzes the esterification of retinol into retinyl esters, an essential reaction for the retinoid homeostasis [77,78] (Figure 1).

Recently, Nakazawa *et al* have studied the effects of nitrofen on the retinoid-signaling pathway in hypoplastic lungs [79]. They demonstrated that *LRAT* was down-regulated causing a shift of retinol from storage to conversion in RA. This suggests that nitrofen disturbs retinoid signaling at an early stage of this pathway rather than by blocking RALDH as mentioned by May et al [61]. Kim *et al* have also confirmed recently that retinyl ester formation by LRAT is a key regulator of retinoid homeostasis in mouse embryogenesis and that, in contrast, the pathway of RA synthesis does not contribute significantly to the regulation of retinoid homeostasis during mammalian development [80]. Interestingly, RA receptors and GATA transcription factors activate the transcription of the human LRAT gene [81].

Chromosome 6

6q23.3: *ALDH8A1 / RALDH4* [MIM606467]

Two reports describe a del(6)(q23) associated with CDH [32]. Lurie suggested that the distal part of 6q may contain a locus whose deletion leads to CDH [32]. Furthermore, Howe *et al.* have shown a balanced t(6;8)(q24;q23) translocation in a CDH fetus [43]. One candidate gene in this region could be the *RALDH4* (Retinal Deshydrogenase 4) encoding a protein belonging to the aldehyde dehydrogenases family of proteins. This protein plays a role in the *in vivo* pathway of 9-cis-retinoic acid biosynthesis (Figure 1). This

enzyme converts 9-cis-retinal into the retinoid X receptor ligand 9-cis-retinoic acid, and has approximately 40-fold higher activity with 9-cis-retinal than with all-trans-retinal.

Chromosome 8

8p23.1: *GATA4* [MIM 600576]

The human *GATA4* gene is located on 8p23.1 where microdeletions are recurrent abnormalities in patients with CDH [32,37,82,83]. Faivre *et al* suggested that cases of a combination of CDH and cardiac defect should be analyzed for the presence of 8p23.1 deletion [42]. GATA4 is a zinc-finger transcription factor expressed in mesenchymal cells of the developing diaphragm, lung and heart. The expression and activity of GATA4 are influenced by retinoids [84,85]. Jay *et al.* described a novel mouse model of CDH based on heterozygosity of a GATA4 deletion mutation [86]. This $GATA4^{+/\Delta ex2}$ mouse developed midline diaphragmatic hernia, dilated distal airways, thickened pulmonary mesenchyme and cardiac malformations. Recently, somatic mutations have been detected in *GATA4* and other "cardiac" transcription factors in the hearts of patients who died of congenital heart disease [87]. Thus, somatic mutations that arise during cardiogenesis may be a novel molecular cause of congenital heart disease and it is conceivable that somatic mutations in *GATA4* might contribute to the pathogenesis of CDH.

8q23.1: *FOG2 / ZFPM2* [MIM 603693]

Three patients with CDH and 8q deletions have been reported in Holder's review [23]. Temple *et al.* described two CDH patients with a balanced translocation involving the 8q22.3 region and CDH [88]. Howe *et al.* have also shown a balanced t(6;8)(q24;q23) in a patient with CDH [43]. *FOG2* (Friend of *GATA2*) is located at the proximal region of the 8q23.1 band flanking the 8q22.3 band. We also suggest that the truncation of *FOG2* or a positional effect affecting the transcription regulatory of this gene could be responsible for a CDH in these 3 patients. FOG2 is a multi-zinc finger transcriptional protein that binds to members of the family of transcription factors as GATA4. *FOG2* is expressed in mesodermal tissues including pulmonary mesenchyme, mesothelium and pleuroperitoneal fold tissue [89]. It has been demonstrated that this protein can activate or down-regulate expression of GATA-target genes via the

formation of a heterodimer with transcription factors of the GATA family (GATA4, GATA5 and GATA6), suggesting different modulation depending on the cell and promoter context. Huggins *et al* demonstrate that FOG-2 can serve as a corepressor protein for both COUP-TFII and GATA4 proteins [90]. Jay et al suggest that a concerted action of FOG2 and GATA4 is required to regulate mesenchymal cell function in the developing diaphragm and lung [86]. In a screen of fetal mice carrying chemically induced genetic mutations, Ackerman *et al* found that a mutation in the gene *FOG2* causes abnormal diaphragm development and pulmonary hypoplasia [91]. Based on this result, the authors identified a *de novo R112X* heterozygous mutation in an infant who died shortly after birth with diaphragmatic defect and severe pulmonary hypoplasia [91]. More recently, Bleyl *et al* have identified two novel sequence alterations in *FOG2* in two patients with isolated CDH, reinforcing the hypothesis that FOG2 is critical for normal development of the diaphragm [92].

Chromosome 12

CDH is one of the most frequent abnormalities described in tetrasomy 12p cases, also known as Pallister-Killian syndrome. Likewise, Tonks *et al.* described a t(3;12)(q21.1;p13.3) balanced translocation associated with CDH [28]. We identified two candidate genes related to retinoids on the chromosome 12p13.

12p13.31: *RBP5 / CRBPIII* [MIM 611866]

RBP5 (Retinol Binding Protein 5) is a new family member of RBPs and is predominantly expressed in the liver [93]. RBP5 binds *all-trans* retinol with a specific interaction similar to that observed in the retinol-RBP1 complex. RBP5 is a direct target of PPAR-gamma, a member of the peroxisome proliferator-activated receptor (PPAR, MIM 601487) subfamily of nuclear receptors. PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various genes [94]. Alteration of 9-cis RA generation could modify the PPAR/RXR activation and therefore RBP5 expression. It is not known to date whether the *RBP5* gene is expressed and whether it plays a role during embryogenesis.

12p13.1: *RAIG1 / RAI3* [MIM 604138]

The *RAIG1* (Retinoic Acid-Inductible Gene 1) gene encodes a member of the type 3 G protein-coupling receptor family, characterized by the "7-transmembrane domain motif" signature [95]. This G-protein coupled receptor could be involved in modulating differentiation and maintaining homeostasis of epithelial cells. The comparable expression level in fetal lung and kidney with adult tissues suggests a possible role in embryonic development and maturation of these organs. *RAIG1* expression is induced by all-trans-retinoic acid via its receptors [96]. The encoded protein may be involved in the interaction between retinoid acid and G protein cellular signaling pathways.

Chromosome 15

15q24.1: *STRA6* [MIM 610745]

Sharp *et al.* described a patient with a *de novo* 15q24 microdeletion associated with diaphragmatic hernia [97]. Aviram-Goldring *et al.* studied a family in which two fetuses had CDH associated with an apparently balanced t(5;15)(p15.3;q24) also present in the mother and in a normal child, suggesting that the CDH in these fetuses may have been caused by a cryptic imbalance at one of the breakpoints during meiosis [98]. More recently, Van Esch *et al* described a 15q24 microdeletion of 3.1 Mb including the STRA6 gene in a patient with severe mental retardation, facial dysmorphisms and CDH [99]. *STRA6* encodes a specific receptor for RBP4-retinol located on cell membranes in the target tissues (Fig 1). It removes the retinol from RBP4 and mediates retinol uptake by cells [100-102]. The transcription of *STRA6* is directly regulated by RA levels. During embryogenesis, *STRA6* is expressed in respiratory mesenchyme and in respiratory/bronchial epithelium [103]. Consistent with various roles of vitamin A and the wide tissue expression pattern of *STRA6*, mutations in *STRA6* are associated with severe pathological phenotypes in humans. CDH is an important component of the phenotype observed in cases of *STRA6* mutations [15,104].

15q24: *CRABP1* [MIM 180230]

CRABP1 (cellular retinoic acid binding protein 1) belongs to a superfamily of lipid-binding proteins that are thought to act by maintaining tolerable concentrations of intracellular RA as modulators of RA catabolism and as intracellular transporters for RA from the cytoplasm to nuclear receptors [105-107]. CRAPB1 is supposed to play an important role in retinoic acid-mediated differentiation and proliferation processes.

15q26.1-q26.2: *COUP-TFII / NR2F2* [MIM 107773]

A minimally recurrently deleted region has been identified using FISH and aCGH on chromosome 15q26.1-q26.2 in patients with non-isolated CDH [32-34,38,40,108].

COUP-TFII is a transcriptional factor from the steroid/thyroid hormone receptor superfamily. This gene is a nuclear orphan receptor expressed during embryonic development in a variety of tissues, including mesodermal derivatives in the diaphragm, lung and heart [109]. Homozygous tissue-specific deletion of *COUP-TFII* in mice causes posterolateral CDH similar to the Bochdalek-type CDH seen in humans [110].

COUP-TFII appears to be a good candidate in the 15q26 region because i) its expression is regulated by retinoids and ii) COUP-TFII regulates gene transcription by influencing RAR/RXR heterodimerization [111,112]. COUP-TFII is able to sequester RXR in a functionally inactive complex and to reduce the available nuclear concentrations of RXR. Thus, COUP-TFII can act as a repressor of the retinoid pathway, by preventing RAR/RXR heterodimer formation and inhibiting target gene transcription. [112]. In the nitrofen rat model, the repression of retinoid signaling pathway by up-regulation of COUP-TFII may cause hypoplastic lung [18]. This process may be a negative feedback system that precisely balances the transcription of relevant genes during diaphragm development. COUP-TFII has been shown to interact physically with FOG2, implying that these two factors may cooperate during diaphragm morphogenesis [90] (Figure 1).

Recently, Clugston *et al.* have reinforced this hypothesis showing that 15q26 contains a cluster of genes, including COUP-TFII, which are expressed in the developing rodent diaphragm [113].

Taken together, these data suggest that *COUP-TFII* is likely to play a key role in diaphragm development even if no mutations have been found in 73 CDH samples tested by Scott *et al* [38] and in more than 100 samples tested by Slavotinek *et al* [41].

Chromosome X

Xp22.3: *TBL1X* [MIM 300196]

The Xp22.3 region is frequently affected in CDH [23]. This region carries the *TBL1X* (Transducin beta-like 1X) gene which encodes a protein that plays an essential role in transcriptional activation mediated by nuclear receptors [114]. TBL1X is found as a subunit in corepressor SMRT (silencing mediator for retinoid and thyroid receptors) complex along with histone deacetylase 3 protein, known to modulate the nuclear retinoid signaling pathway [115] .

DISCUSSION

A major challenge of CDH research is to characterize genes and signaling pathways that are critical for early mesenchymal cell function during morphogenesis of the diaphragm. Although several genes have been clearly shown to underlie abnormal diaphragm development in mice, few CDH-related mutations have been identified in the corresponding genes in humans. In this review, we focused on genes involved in retinoid metabolism or regulated by retinoids, which are located within chromosomal regions recurrently affected in CDH patients.

The analysis of chromosomal aberrations may help in the mapping of disease loci and isolation of disease genes by positional cloning strategy [46,116]. The principal pitfall in this chromosomal approach is that the localization of breakpoints may not be accurate since it is generally based on standard karyotyping. Recent aCGH technology provides a more precise characterization of chromosomal abnormalities which helps to define the minimal affected region in patients with CDH and identify candidate genes within this region [38,40,41]. *De novo* microdeletions in the regions 1q41-q42, 4p16.3, 8p23.1 and 15q26.1-q26.2 have then been reported. These deleted chromosomal regions may be assumed to contain genes necessary for normal diaphragm development and these genes can subsequently be selected for sequencing in CDH patients. Among the retinoid related genes included in these regions, *COUP-TFII* and *FOG2* were sequenced in CDH patients. Up to date, no mutation could be identified in COUP-TFII [38,41]. A *de novo* mutation and sequence alterations in FOG2 were found in 3 patients, reinforcing the hypothesis that FOG2 is critical in diaphragmatic and lung development in humans. [91,92].

The *STRA6* gene located at chromosome 15q24.1 is also a promising candidate since CDH is an important component of the polymalformative syndrome observed in cases with *STRA6* mutations. Recently, Isken *et al* have shown that STRA6 is essential to maintain embryonic RA homeostasis and that STRA6-dependent transfer of retinol from RBP4 depends on LRAT [117]. LRAT activity is required, like those of STRA6 and RBP4 protein, for uptake of appropriate amounts of retinol into cells. Then, LRAT is also likely to play a role in the development of CDH in individuals with 4q32.1-q31 rearrangements.

Other CDH-associated chromosomal hot spots such as 2q37, 6p25 and 22q11 do not contain genes related to the retinoid pathway. These regions might carry genes involved in pathways regulating differentiation of mesenchymal cells or cell migration, which are important for diaphragm development. For

example, the *COL6A3* gene [MIM 120250] is located on chromosome 2q37, a region frequently deleted in CDH. Impaired formation of the extracellular matrix, caused by disruptions in either collagen or elastic fibers can lead to developmental defects in a wide range of organs including the diaphragm. Of note, CDH has been linked to several subtypes of Ehlers-Danlos syndrome (EDS) caused by mutations in genes belonging to the collagen family (Table 1), which is expressed during embryonic development in several organs [17]. In the same way, the 6p25 region repeatedly deleted in CDH patients contains the *FOXF2* gene (forkhead box F2), one of the human homologues of the *Drosophila melanogaster* transcription factor forkhead, which could be a good candidate [23,38]. *FOXF2* is expressed in lung and placenta and was shown to activate transcription of several lung-specific genes [118,119].

Systematic screening for mutations in CDH patients has been reported only for *WT1*, *COUP-TFII* and *FOG2*. The same approach could be used to search for mutations in related genes from the RA metabolic and molecular signaling pathway. In addition, a simultaneous analysis of several loci by QMPSF (Quantitative Multiplex PCR of Short fluorescent Fragments) in a cohort of CDH patients may be used to estimate the frequency of microdeletions or microduplications of candidate genes, which may help to establish their role in CDH etiology. The identification of CDH-related genes and pathways affecting normal diaphragm and lung development will contribute to the understanding of the pathophysiology of this severe embryopathy. Given the substantial mortality and morbidity associated with this developmental abnormality, advances in this area are critical.

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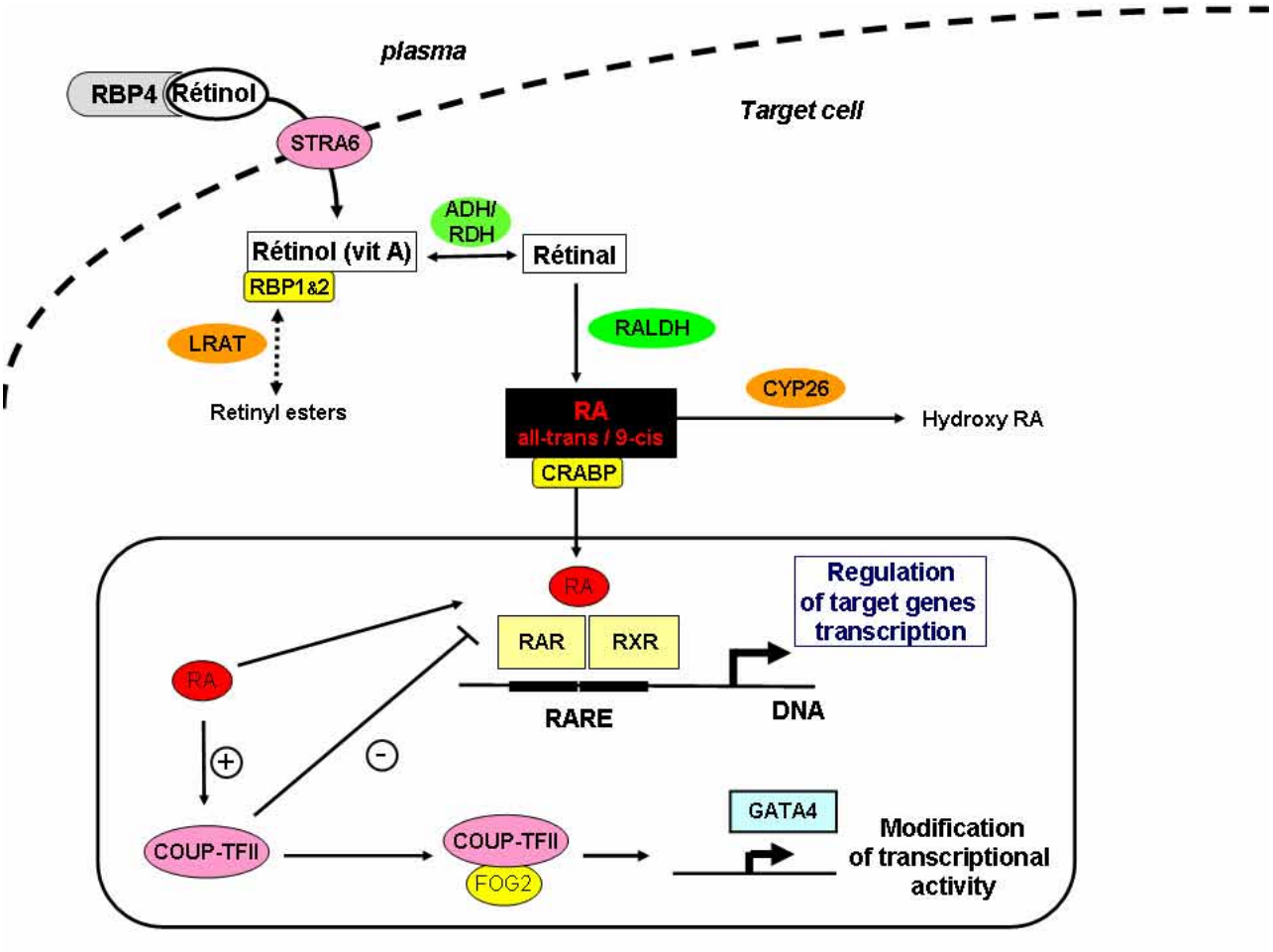
FIGURE LEGEND

Figure 1: A conceptual model describing RA synthesis and signaling in target cells

Retinol transported in the plasma bound RBP4 (plasma retinol-binding protein). STRA6 binds RBP4, removes the retinol from RBP4 and transports it across the plasma membrane, where it can be metabolized. Within the target cell, retinol binds to cellular retinol binding protein (CRBP, gene names RBP1 and RBP2) that regulates the cellular metabolism of retinol by presenting it either to alcohol or retinol dehydrogenases (ADHs / RDHs) for conversion to retinal or to LRAT (lecithin: retinol acyltransferase) that esterifies the retinol to retinyl esters. Retinal is then oxyded to RA by retinal dehydrogenase (RALDH). Cellular retinoic-acid-binding protein (CRABP) assists RA entry into the nucleus and RA exerts its biological effects through binding to nuclear receptors RARs and RXRs. Excess of RA is catabolized in the cytoplasm by the CYP26 class of P450 enzymes.

In the nuclei, RA binds to RAR and RXR, which themselves heterodimerize and bind to short DNA sequence: the retinoic acid-response element (RARE). This binding activates the transcription of many target genes.

COUP-TFII can act as a repressor of this pathway by directly sequestering RXR, thereby preventing the formation of RAR/RXR heterodimer and inhibiting gene transcription. COUP-TFII interacts with FOG2 that modulates the transcriptional activity of GATA4 proteins, which a transcription factor playing an important role in early embryogenesis.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Target cell
RBP4
Rétinol
STRA6
ADH/
RDH
Rétinol (vit A)
RBP1&2
Rétinal
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LRAT
Retinyl esters
RA
all-trans / 9-cis
CYP26
Hydroxy RA
CRABP
RA
RAR
RXR
Regulation
of target genes
transcription
RA
RARE
DNA
COUP-TFII
COUP-TFII
FOG2
GATA4
Modification
of transcriptional
activity

Figure 1

Table 1: Monogenic syndromes in which CDH commonly occurs

Syndrome	OMIM	Gene	Chromosomal location
Simpson-Golabi-Behmel	312870	*GPC3* (glypican-3)	Xq26.1
Denys-Drash	194080	*WT1* (Wilms tumor 1)	11p13
Donnai-Barrow	222448	*LRP2* (low density lipoprotein-related protein 2)	2q31.1
spondylocostal dysostosis	277300	*DLL3* (delta-like-3)*	19q13.2
Matthew-Wood	601186	*STRA6* (stimulated by retinoic acid gene 6 homolog)	15q24.1
craniofrontal dysplasia	304110	*EFNB1* (Ephrin B1)	Xq12
Cornelia de Lange	122470	*NIPBL* (nipped-B-like)	5p13.1
Marfan	154700	*FBN1* (fibrillin 1)	15q21.1
Ehlers-Danlos type IV and type VII	130050 130060	*COL3A1* (collagen type III), *COL1A1* and *COL1A2*	2q31, 17q21-q22, 7q22.1

* most commonly mutated gene

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Birth Defects Research (Part A) 00:000–000 (2009)

Brief Report

Fetal Skin Fibroblasts: A Cell Model for Studying the Retinoid Pathway in Congenital Diaphragmatic Hernia

AQ1 **Carole Goumy,[1,2*] Karen Coste,[2] Geoffroy Marceau,[2] Laetitia Gouas,[1] Andreï Tchirkov,[1] Philippe Vago,[1] Denis Gallot,[2] and Vincent Sapin[2]**

AQ2 [1]Clermont Université, UFR Médecine, Histologie Embryologie Cytogénétique, Clermont-Ferrand, France; CHU Clermont-Ferrand, Cytogénétique Médicale, Clermont-Ferrand, France
[2]Clermont Université, UFR Médecine, Clermont-Ferrand, France.

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BACKGROUND: Although there is strong evidence that genetic factors play a pathogenic role in congenital diaphragmatic hernia (CDH), few causal genes have been identified in humans. A number of studies, essentially in animal models, have suggested that disruption of the retinoid signaling pathway plays a major role in the pathogenesis of CDH. Our hypothesis is that human fetal skin fibroblasts express some metabolic and molecular actors of the retinoid pathway and that they offer convenient cellular material for investigating the molecular retinoid pathway defects associated with CDH. **METHODS:** We first established the expression of receptors, enzymes and binding proteins involved in the retinoic acid (RA) pathway in non-CDH fetal skin fibroblasts using RT-PCR and immunocytochemistry approaches. We then studied the expression of these genes in skin fibroblasts from seven fetuses with isolated and nonisolated CDH. **RESULTS:** Fetal skin fibroblasts expressed enzymes involved in RA metabolism as well as nuclear receptors for signal transduction. Basal levels of retinoic acid receptor, retinaldehyde dehydrogenase 2, and CYP26 (cytochrome P450 RAI) expression were altered in two of seven fetuses. Interestingly, these genes were previously described as abnormally expressed in CDH physiopathology. **CONCLUSION:** Our results suggest that human fetal skin fibroblasts could be useful for studying retinoid signaling pathway disruption in the context of CDH. Our proposal is strengthened by the fact that we identified CDH fetuses for which molecular and metabolic actors of retinoid pathway were not detected. *Birth Defects Research (Part A) 00:000–000, 2009.* © 2009 Wiley-Liss, Inc.

Key words: retinoid pathway; congenital diaphragmatic hernia; fetal skin fibroblasts

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a severe developmental anomaly that affects one in 3000 live births and is associated with high mortality (Colvin et al., 2005; Yang et al., 2006; Gallot et al., 2007). However, the molecular pathogenesis of CDH remains poorly understood. Several theories have been proposed on the main embryologic events leading to CDH, including abnormalities in lung development, failure of the pleuroperitoneal canals to close, defective myoblast formation, and abnormal phrenic nerve development (Iritani, 1984; Thebaud et al., 1999). The identification of pleuroperitoneal fold (PPF) defects in nitrofen-exposed rat fetuses, newborn rats bred on a vitamin A–deficient diet, and mutant mice with functionally inactive *WT1* or *COUP-TFII* genes strongly suggested that the embryogenesis of this structure was a major focal point for elucidating the pathogenesis of CDH (You et al., 2005; Clugston et al., 2006). Moreover, Clugston et al. (2007) suggest that the defective component of the PPF was the mesenchyme (and not the muscular substratum), which becomes unable to provide a complete foundation for the formation of diaphragmatic musculature between the fifth and seventh week of gestation in humans.

Abnormalities in the retinoid signaling pathway and its downstream molecular and cellular target sequences have been hypothesized to lead to the development of

*Correspondence to: Carole Goumy, Cytogénétique Médicale, Faculté de Médecine – BP 38, 63001 Clermont-Ferrand Cedex – France.
E-mail: cgoumy@chu-clermontferrand.fr
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CDH, because of the fundamental roles played by these active derivates of vitamin A during lung development (Greer et al., 2003). Retinoids play a crucial role in regulating gene expression through interactions with nuclear receptors, retinoic acid receptors (RARs), and retinoid X receptors (RXRs) during mammalian embryonic development. The retinoid family is designated as vitamin A (all-*trans* retinol) and its various metabolites, with retinoic acid (RA) being its most active form. The balance between active retinoid synthesis and degradation determines RA concentrations in target cells. RA is generated by a series of oxidative reactions that convert retinol to retinaldehyde and ultimately to the active forms (Zhao et al., 1996). Several enzymes have been shown to catalyze these reactions, but retinaldehyde dehydrogenase 2 (RALDH2) has a prominent role in generating RA (Niederreither et al., 1999). Because of their hydrophobic characteristics, retinol, retinaldehyde and/or retinoic acid had to be solubilized intracellularly by specific binding proteins that also possess other physiologic roles. Indeed, cellular retinol-binding proteins (CRBPs) were able to bind the retinol delivered by the blood retinol-binding protein (RBP) via capture by the transmembrane receptor stimulated by retinoic acid 6 (STRA6). They also determine the metabolic fates of intracellular retinol: retinol activation into RA or conversion into retinyl esters for storage. Cellular retinoic acid-binding proteins (CRABPs) assist RA entry into the nucleus where RA exerts its biologic effects by binding to RAR and RXR nuclear receptors. CRABPs also present excess RA for degradation to protect RA-sensitive cells from abnormally high RA levels. P450RAI (CYP26A1, B1, and C1) is an RA-inducible, RA-metabolizing enzyme of the cytochrome P450 family. It converts RA into several hydroxylated products presenting null or limited effects in terms of target gene regulation (White et al., 1996; Fujii et al., 1997).

Imbalances in the concentrations of functional retinoid metabolites could lead to abnormal retinoid signaling as abnormal expression, structure or activity profiles of binding proteins, nuclear receptors, or retinoid-metabolizing enzymes. A large body of data strongly suggests that a retinoid signaling pathway disruption could lead to CDH, although the precise mechanisms underlying this event remain unclear (Greer et al., 2003; Gallot et al., 2005). The first evidence linking retinoids with CDH found a high incidence of CDH in the offspring of rats fed vitamin A–deficient diets during gestation (Anderson, 1941). Moreover, the mouse nitrofen toxic model of diaphragmatic hernia showed that nitrofen inhibits RALDH2 and that antenatal administration of vitamin A reduced the incidence of CDH and restored lung maturation (Thebaud et al., 1999; Mey et al., 2003; Babiuk et al., 2004). Nitrofen-induced alteration of RA production was also recently demonstrated to induce abnormal diaphragm development (Clugston et al., 2009). Finally, numerous malformations, including CDH, have been reported in RARα/RARβ double-knockout mouse models (Mendelsohn et al., 1994). However, few human studies have pinpointed retinoid signaling pathway disruption in the pathogenesis of CDH. One small clinical study revealed that infants with CDH had lower plasma vitamin A and RBP levels than did healthy infants (Major et al., 1998). It was recently shown that mutations in human *STRA6* cause a malformative syndrome associating CDH, anophthalmia, heart defects, lung hypoplasia, and mental retardation. To date, this is the only example of a retinoid signaling pathway gene mutation causing developmental abnormalities in humans (Golzio et al., 2007; Pasutto et al., 2007).

In animal CDH models, lung and diaphragm cells have been used extensively to investigate the expression pattern of RA-pathway actors (Nakazawa et al., 2007; Clugston et al., 2008), whereas in human CDH, only one study used postmortem lung samples (Rajatapiti et al., 2006). The use of such human tissue may sometimes give only limited, high-quality, intact RNA, because of substantial delays between death and sampling. To addess this problem, we propose using fetal skin fibroblasts as a model for studying the expression of the different actors of the retinoid pathway. The skin is an extremely retinoid-responsive tissue, and skin fibroblasts have the same mesoblastic origin as the mesenchymal component of the PPF (Ramos-e-Silva et al., 2001; Clugston and Greer, 2007).

Our hypothesis is that fetal skin fibroblasts express metabolic and molecular actors of the retinoid pathway, making them a convenient cellular material for investigating molecular retinoid pathway defects associated with CDH. We used qualitative RT-PCR and immunocytochemical analysis to detect the mRNA and proteins of the retinoid pathway actors in non-CDH fetal skin fibroblasts and to assess their expression in skin fibroblasts of fetuses with CDH.

MATERIALS AND METHODS

Tissue Collections

Ten fetal skin biopsies (seven CDH and three non-CDH) were obtained after parents gave informed consent in accordance with the Declaration of Helsinki. Non-CDH fetuses were obtained after termination of pregnancy for complicated midtrimester rupture of membranes at 15, 24, and 27 weeks' gestation. All CDH fetuses were terminated except cases 2 and 4, who died immediately after birth despite intensive neonatal management (Table 1). Four of the T1
seven CDH fetuses had an isolated CDH (i.e., two left-sided [fetuses 1 and 2] and two right-sided [fetuses 3 and 4]). One had a cystic hygroma and a left sided-CDH (fetus 5) and two had multiple malformations with chromosomal aneuploidy: one with Pallister-Killian syndrome with 100% tetrasomic 12p cells after primary culture (fetus 6) and one with trisomy 18 (fetus 7). Gestational ages for CDH fetuses range from 16 to 40 weeks (Table 1). The research project was approved by the Institutional Regional Ethics Committee (CPP6 Sud-Est). AQ3

Cell Cultures

Fetal skin fibroblasts were cultured with HAMF10 medium supplemented with 2% ultroser, 5% fetal calf serum (charcoal-stripped to delete all endogenous retinoids), 1% L-glutamine, and 1% penicillin-streptomycin. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C. Medium was routinely changed every 3 to 4 days.

RNA Extraction and RT-PCR

Total RNA was extracted from cultured fibroblasts after the first trypsinization using Trizol (Invitrogen, Cergy-Pontoise, France). The cDNA was generated using a superscript First-Strand Synthesis System for RT-PCR (Gibco-BRL, Cergy-Pontoise, France). RNA quantity was determined by spectrophotometric measurement at 260 and 280

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Table 1
Characteristics of CDH Fetuses

Number	Side	Association	Fetal age (weeks)
CDH1	Left	Isolated	25
CDH2	Left	Isolated	40
CDH3	Right	Isolated	35
CDH4	Right	Isolated	40
CDH5	Left	Cystic hygroma	16
CDH6	Left	Tetrasomy 12p	27
CDH7	Left	Trisomy 18	24

CDH, congenital diaphragmatic hernia.

nm (ratio with protein). RNA quality was analyzed by RNA/protein ratio (260/280 nm) and gel electrophoresis (1% agarose) to observe the presence of intact 28S and 18S RNA bands. Specific oligonucleotide primers were generated using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_wwwcgi) based on the published full-length human RNA sequences of each specific gene and designed to avoid genomic DNA amplification. All the primers were first checked for their specificity to amplify defined mRNA regions using human tissue already reported to express these genes (positive control), as previously established by Marceau et al. (2006). PCR amplification was performed in an Eppendorf Mastercycler, using 50 ng of total cDNA per reaction, according to the following program: initial denaturation step at 95°C for 5 minutes, followed by denaturation at 95°C for 45 seconds, annealing at 59°C for 45 seconds, and extension at 72°C for 60 seconds (36 cycles), and terminated by a final extension of 72°C for 7 minutes. Amplification of the housekeeping gene acidic ribosomal phosphoprotein P0 (36B4) was used as positive control. A negative control for amplimer contamination was established using a complete PCR mix without cDNA.

Immunocytologic Experiments

Fetal skin fibroblasts grown in Laboratory-Lek culture chambers were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) at 25°C for 10 minutes, rinsed three times with PBS, incubated at 25°C for 10 minutes in H_2O_2 (quenching endogen peroxidases), and incubated in PBS with 3% bovine serum albumin (Sigma-Aldrich, Saint-Quentin-Fallavier, France) at 25°C for 30 minutes. Cells and tissues were incubated overnight at 4°C in the presence of RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ (respectively, Santa-Cruz (sc) 551,

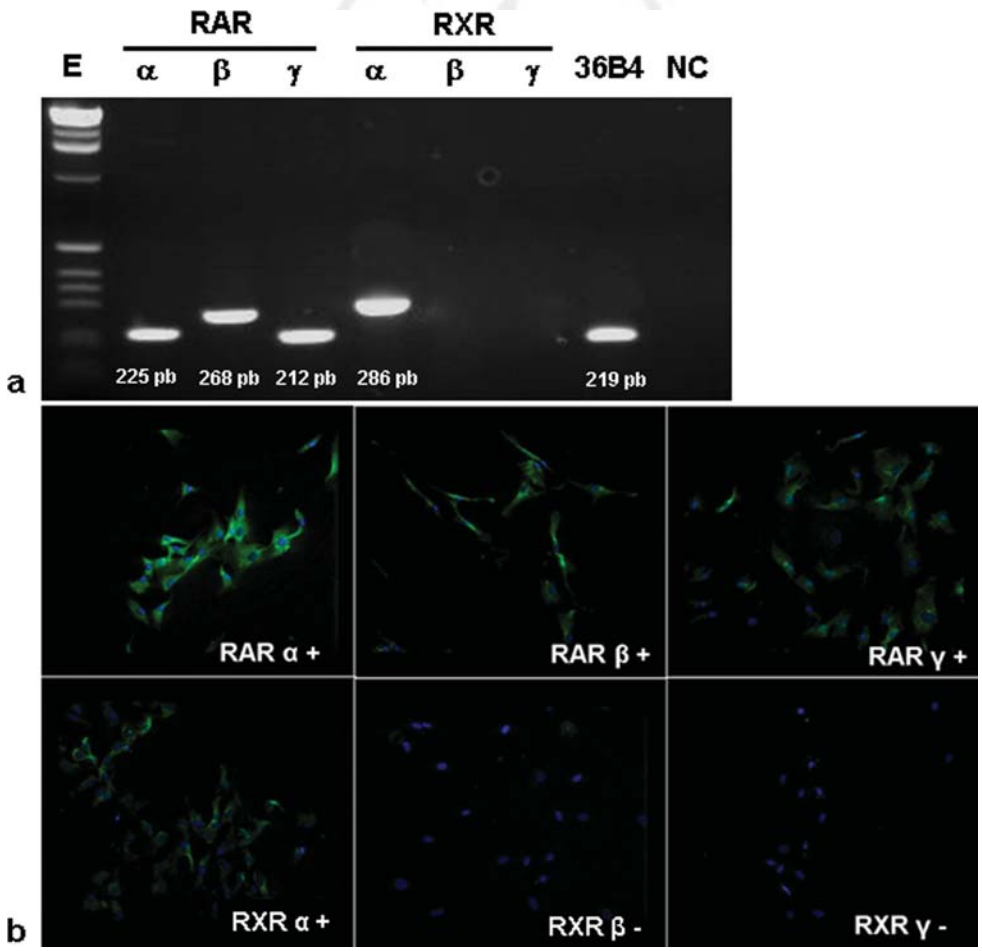

Figure 1. Expression pattern of nuclear retinoid receptors (RARs and RXRs) in cultured fibroblasts of one non-CDH fetus. Expression patterns were established at the mRNA level (**a**) and the protein level (**b**). (**b**) Positive-label retinoid nuclear receptors are shown in green (magnification, ×400). Blue labeling is nuclear localization. E, molecular weight; bp, base pairs; NC, negative control.

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Table 2
RT-PCR Expression Patterns of Nuclear Receptors in Fetal Fibroblasts of Non-CDH and CDH Fetuses

	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
Non−CDH (n=3)	+	+	+	+	−	−
CDH1	+	+	+	+	−	−
CDH2	+	+	+	+	−	−
CDH3	+	+	+	+	−	−
CDH4	+	+	+	+	−	−
CDH5	+	+	+	+	−	−
CDH6	−	−	−	+	−	−
CDH7	+	−	+	+	−	−

CDH, congenital diaphragmatic hernia; RAR, retinoic acid receptor; RXR, retinoid X receptors.

552, 550, 553, 831, and 555) certified rabbit polyclonal primary antibodies (1/200 in PBS) (Tebu, Le Perray-en-Yvelines, France). This step was followed by three PBS washes, 1 hour incubation in the presence of a secondary goat HRP anti-rabbit antibody (Interchim, Montluçon, France) at room temperature, and an additional three PBS washes. The samples were then examined after 4,6-diamino-2-phenylindole (DAPI) nuclear staining (5 minutes, dilution in PBS 1/500) and mounting in an Vectashield aqueous mounting fluid (Vector, Burlingame, CA) under a Zeiss Axioplan epifluorescence microscope. For negative controls, sections were incubated with normal rabbit IgG instead of anti-RARs or anti-RXRs.

RESULTS

Expression Patterns of RAR and RXR Nuclear Receptors In Non-CDH and CDH Fetal Skin Fibroblasts

The three RARs and the three RXRs showed similar transcript expression patterns in three non-CDH fetal fibroblasts. They all presented positive expression for RARα, RARβ, RARγ and RXRα whereas mRNA for RXRβ and RXRγ was not detectable (Fig. 1a). The protein expression of these receptors studied by immunocytochemical analysis established that RARα, RAR β, RARγ and RXRα proteins were expressed in fetal skin fibroblasts, whereas RXRβ and RXRγ were not detected, in accordance with mRNA results (Fig. 1b).

Five CDH fetal fibroblasts (CDH1 to CDH4 with isolated CDH and CDH5 with cystic hygroma) showed similar RAR/RXR expression patterns to non-CDH fibroblasts in terms of transcripts and proteins. In fetus CDH 6 (tetrasomy 12p), none of the RAR subunits were detected, whereas the RXR expression patterns were similar to those observed in non-CDH fetuses (Table 2). The particular pattern was confirmed at the protein level by immunocytochemistry showing no immunostaining for these three nuclear receptors. In fetus CDH7 (trisomy 18), mRNA and protein for RARβ were not detected (data not shown).

Expression Patterns of Metabolic Retinoid Pathway Actors in Non-CDH and CDH Fetal Skin Fibroblasts

Non-CDH fibroblasts showed positive mRNA expression for ADH3, ADH4, RALDH2, RALDH3, CYP26B1, CRBP1, CRABP1, and CRABP2. RALDH1 expression was detected in two of the three non-CDH fetuses (Table 3). In contrast, the mRNA of ADH1A, RALDH4, CYP26A1, CYP26C1, and CRBP2 were never detected. Except for fetuses CDH2 and CDH6, all CDH skin fibroblasts showed similar expression patterns to non-CDH fibroblasts. In fetus CDH6 (tetrasomy 12p), neither CRABP1 nor the three CYP26 family members were detected, but CRABP2 was still present. In fetus CDH2, RALDH2 was not detected, but RALDH3 was still present. CDH fibroblasts showed variable RALDH1 expression.

DISCUSSION

This study is the first to establish the expression of the metabolic and molecular actors involved in the retinoid signaling pathway on non-CDH and CDH fetal skin fibroblasts. These findings represent promising new tools for providing insight into the physiopathology of CDH and the links between retinoids and CDH in humans. Whereas several animal datasets have clearly implicated the retinoids in CDH, only one human study has focused on associations between CDH and nuclear retinoid pathways (Rajatapiti et al., 2006). CDH is a relatively rare malformation, making it difficult to collect enough fetal material from defective tissues to perform gene expression studies. Furthermore, recovering high-quality intact RNA from postmortem tissue is a major difficulty, because RNAs are unstable molecules prone to rapid degradation by ribonucleases. The availability of postmortem tissue is often associated with substantial delay between fetal death and biopsy. This difficulty prompted us to use fetal skin fibroblasts based on the rationale that skin biopsy is easily performed at necropsy. In addition, skin fibroblasts are relatively easy to grow, can be maintained in culture as primary cell lines without significant phenotypic change for many generations, and are now well established as routine laboratory procedure. Being undifferentiated, fibroblasts express numerous molecules of various biologic systems, provide homogeneous material, and yield reproducible and reliable data because the cell can be continuously grown in culture. Furthermore, it has been well illustrated that the skin is an extremely retinoid-responsive tissue, and skin fibroblasts may have the same mesoblastic origin as the septum transversum, which cause the major parts of the diaphragm, and as the mesenchymal component of the PPF that was presumed defective in CDH (Ramos-e-Silva et al., 2001; Clugston et al., 2007). The main limitation of this cell model is that the initial perturbation associated with CDH occurs at 5 to 7 weeks' gestation, whereas fetal biopsies have been performed at a much later developmental stages, when CDH has already been diagnosed.

Our study clearly shows that cultured fetal human skin fibroblasts express RARα, RAR β, RARγ, and RXRα, but neither RXRβ nor RXRγ at either the mRNA or protein level. It has previously been shown that human postnatal dermal fibroblasts expressed RARα, RARγ RXRα, and RXRβ, but not RARβ and RXRγ (Tsou et al., 1994, 1997). These differences established for retinoid nuclear receptor expressions were already noted for other tissues between the developmental and postdevelopmental stages (Mark et al., 2009). Nevertheless, both steps may involve heterodimerization between RARs and RXRs, leading to a functional molecular stimulation by retinoid ligands. Our data demonstrate that RARβ was only

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Table 3
RT–PCR Expression Patterns of Retinol Metabolic Enzymes and Binding Proteins in Fetal Fibroblasts of Non–CDH and CDH Fetuses

	ADH1	ADH3	ADH4	RALDH1	RALDH2	RALDH3	RALDH4	CYP26A1	CYP26B1	CYP26C1	CRBP1	CRBP2	CRABP1	CRABP2
Non–CDH (n = 3)	−	+	+	+/−	+	+	−	−	+	−	+	−	+	+
CDH1	−	+	+	+	+	+	−	−	+	−	+	−	+	+
CDH2	−	+	+	−	−	+	−	−	+	−	+	−	+	+
CDH3	−	+	+	+	+	+	−	−	+	−	+	−	+	+
CDH4	−	+	+	+	+	+	−	−	+	−	+	−	+	+
CDH5	−	+	+	+	+	+	−	−	+	−	+	−	−	+
CDH6	−	+	+	−	+	+	−	−	−	−	+	−	+	+
CDH7	−	+	+	−	+	+	−	−	+	−	+	−	+	+

CDH, congenital diaphragmatic hernia.

expressed in fetal fibroblasts. Similarly, Kimura et al. (2002) have shown that RARβ was detected in distal mesenchymal cells of the fetal lung but not the adult lung. It is equally well known that RARβ expression is strongly retinoid-dependant (Borel et al., 2009). This differential expression could be explained by a modulation of functional retinoic acid concentrations in these cells during the prenatal and postnatal stages. To the best of our knowledge, only one gene expression study of retinoic receptors in the CDH fetus lung has been reported to date. It showed that the expression patterns of all RARs and RXRs were similar to those observed in normal controls (Rajatapiti et al., 2006). Our results confirmed this report, because the majority of the CDH fetuses presented the same expression patterns as non-CDH fetuses. In the fibroblasts of the fetus with trisomy 18, we did not detect mRNA for RARβ, probably for the reason mentioned previously. More interestingly, in the fetus with homogene tetrasomy 12p, none of the three RAR subunits were detected in fetal skin fibroblasts. As CDH has been reported in RARα/RARβ double-knockout mouse models, it is tempting to speculate that the presence of CDH in this fetus could be linked to the absence of these RARs (Mendelsohn et al., 1994). Moreover, Clugston et al. (2009) recently showed that the inhibition of RAR signaling with the pan-RAR antagonist BMS493 induced a high degree of CDH with a marked left-right sidedness that was dependent on the timing of drug delivery. To verify this result, we also tested RAR and CYP26 expression in fibroblast cultures from four other CDH fetuses presenting mosaic tetrasomy of chromosome 12p. These four fetal fibroblasts expressed RAR and CYP26 (data not shown). However, the percentage of cells with 12p tetrasomy in these four fetuses ranged from 30 to 70% in primary cultures, whereas primary fibroblast cultures of CDH6 fetus showed a 100% count of abnormal cells. The presence of normal cells expressing RAR and CYP26 could be an explanation for this discrepancy.

Regarding enzymes and intracellular binding proteins involved in the synthesis and degradation of RA, we showed that at least one member of each enzymatic step (conversion of retinol into RA, degradation of RA) and binding proteins was detected in non-CDH fetal fibroblasts. This finding suggests that these cells possess the complete metabolic capacities of retinoids. Indeed, our preliminary data also established that these fetal skin fibroblasts express the metabolic enzymes implicated in the ''formation/hydrolysis'' of retinyl esters. Analysis of the CDH fetuses found expression patterns globally similar to those observed in non-CDH fetuses. We identified one CDH fetus that did not express RALDH2, a key enzyme for the conversion of retinal in RA. In utero exposure to the nitrofen herbicide, which inhibits the enzymatic activity of RALDH2, was shown to cause CDH and primary lung defects (Mey et al., 2003). Moreover, Clugston et al. (2009) indicates that RALDH2 is responsible for the synthesis of RA in the PPF. Our observation of altered RALDH2 profile in the CDH setting is the first described in humans, suggesting that RALDH2 might be a good candidate for CDH in humans. Further study is needed to screen for RALDH2 mutations in CDH patients. We also noted that RALDH3 is still present in these fetal skin fibroblasts, raising the question of the absence or presence of functional redundancy in terms of RALDH member family. In the tetrasomy 12p fetus fibro-

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blasts, CRABP1 was not expressed but could be replaced by CRAPB2, which was still present. Interestingly, human CDH has already been associated with deletions on the 15q chromosome, which contains CRABP1 (Hengstschläger et al., 2004). In this fetus, none of the three CYP26 were detected, whereas all other fetuses expressed CYP26B1. CYP26B1 is the predominant subtype expressed in the developing murine lung (Abu-Abed et al., 1998; Blomhoff and Blomhoff, 2006). Previous research has demonstrated that CYP26 expression is strongly induced by RA in early mouse and *Xenopus* species embryos with an identifiable conserved retinoic acid responsive element (RARE) in the CYP26 promoter (Loudig et al., 2000). Hence, failure to detect CYP26 expression in the fibroblasts of this fetus could be explained by the fact that none of the three RARs are present to induce its transcription, breaking the RA induction.

In conclusion, we show that fetal skin fibroblasts express enzymes involved in RA metabolism and nuclear receptors for signal transduction. These data lead us to propose that fetal fibroblasts could be used to study the retinoid pathway in CDH fetuses. Our proposal is strengthened by the fact that we identified CDH fetuses for which molecular and metabolic actors of the retinoid pathway (i.e., RARs, RALDH2, and CYP26B1) were not detected. These qualitative results have to be completed by further quantitative analyses at the mRNA and protein levels.

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RESEARCH LETTER

Congenital diaphragmatic hernia and genital anomalies: Emanuel syndrome

A. S. Gremeau[1], K. Coste[2,3], P. Blanc[3], C. Goumy[3,4], C. Francannet[2], P. J. Dechelotte[3,5], P. Vago[4], H. Laurichesse-Delmas[1,3], A. Labbe[2,3], D. Lemery[1,3], V. Sapin[3] and D. Gallot[1,3]*

[1]*Maternal Foetal Medicine Unit, CHU Clermont-Ferrand, France*
[2]*Department of Paediatrics, CHU Clermont-Ferrand, France*
[3]*UMR CNRS 6247 GReD, Clermont Université, France*
[4]*Laboratory of Cytogenetics, CHU Clermont-Ferrand, France*
[5]*Laboratory of Pathology, CHU Clermont-Ferrand, France*

KEY WORDS: congenital diaphragmatic hernia; genital anomalies; Emanuel syndrome; foetal ultrasound; foetal imaging; general cytogenetics; prenatal cytogenetics; foetal and placental pathology

A 28-year-old patient, gravida 7 para 3, was referred to our foetal medicine unit for left-sided congenital diaphragmatic hernia (CDH) diagnosed at 33 weeks of gestation. Ultrasound scan confirmed left-sided CDH with polyhydramnios, major mediastinal shift, intrathoracic stomach, intrathoracic liver and lung-to-head ratio 0.85. An associated anomaly was a genital malformation (micropenis) (Figure 1). Foetal karyotyping revealed 47,XY,+der(22)t(11;22)(q23;q11). Maternal karyotype was 46,XX,t(11;22)(q23;q11). Extensive genetic counselling was offered to the parents, who decided not to terminate pregnancy. The patient delivered vaginally at 37 weeks and 2 days. The baby was immediately intubated and admitted to the neonatal intensive care unit. Clinical and radiological examination confirmed left-sided CDH associated with micropenis and revealed a left pre-auricular tag, broad forehead, high-arched palate, low-set ears, a hypoplastic posterior corpus callosum and 13 pairs of ribs. Surgery was performed on Day 2 and confirmed agenesis of the left part of the diaphragm. Evolution showed persistent pulmonary hypertension, intrahepatic cholestasis, necrotizing enterocolitis and sepsis. The baby died on Day 32 from multiple organ dysfunction.

The frequency of additional major malformations present in babies with CDH was 39% in one meta-analysis (Skari *et al*., 2000). The accompanying anomalies most frequently affect the cardiovascular system (up to 50%), genitourinary system (23%), gastrointestinal system (14%) and central nervous system (10%). The association of CDH with a single genital anomaly is very rare except for cryptorchidism, which can be observed in up to 30% of male CDH foetuses. Cryptorchidism may be induced by decreased intra-abdominal pressure and should probably not be considered an independent malformation. Thus, genital anomaly in the context of CDH is usually associated with other extragenital signs. These signs are sometimes difficult to detect prenatally as illustrated by our case report. Table 1 lists the five syndromes in which the association of CDH and genital anomaly in men is most frequent. This association has also been reported occasionally in many genetic syndromes such as CHARGE, Smith-Lemli-Opitz, Simpson-Golabi-Behmel, Kabuki and VACTERL.

Emanuel syndrome is characterised by severe mental retardation, microcephaly, failure to thrive, pre-auricular tag or sinus, ear anomalies, cleft or high-arched palate (50% of cases), micrognathia, kidney abnormalities such as renal agenesis or hypoplasia (30% of cases), congenital heart defects (60% of cases) anal atresia (20% of cases), diaphragmatic hernia and genital abnormalities in men (cryptorchidism, small scrotum, micropenis) (Zackai and Emanuel, 1980). This syndrome is caused by a chromosome imbalance consisting of either a derivative chromosome 22 [der(22)] as a supernumerary chromosome with the following karyotype: 47,XX/XY,+der(22)t(11;22)(q23;q11), or more rarely a balanced (11;22) translocation associated with the supernumerary derivative chromosome. The supernumerary der(22) chromosome is easily identified by routine G-band analysis at the 500 to 550 band level (Zackai and Emanuel, 1980). It results from almost identical breakpoints on both 11q23 and 22q11 (Kurahashi and Emanuel, 2001). Chorionic villous sampling or amniocentesis are equally sensitive in detecting the supernumerary der(22). In more than 99% of cases, one of the parents of a proband with Emanuel syndrome is a balanced carrier of a t(11;22)(q23;q11.2) and is phenotypically normal (Zackai and Emanuel, 1980). This translocation leads to Emanuel syndrome as a result of 3 : 1 meiotic disjunction. Over 100 individuals with supernumerary der(22) have been described in the

*Correspondence to: D. Gallot, Unité de Médecine Fœtale, CHU, Maternité de l'Hôtel-Dieu, boulevard Léon-Malfreyt, 63058 Clermont-Ferrand cedex, France.
E-mail: dgallot@chu-clermontferrand.fr

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Figure 1—Micropenis in a male foetus with Emanuel syndrome at 33 weeks of gestation

Table 1—Association of congenital diaphragmatic hernia (CDH) and genital anomaly in men: the most frequent syndromes

Syndrome	Gene involved/ cytogenetics	Prevalence of CDH	Prevalence of genital anomalies in men	Spectrum of genital anomalies in men
Emanuel (Zackai and Emanuel, 1980)	der(22)t(11;22)	Occasional	30–60%	Cryptorchidism*, small scrotum, micropenis
Fryns (Slavotinek, 2004)	Unknown	>80%	10–20%	Cryptorchidism*, shawl scrotum, micropenis, ambiguous genitalia, hypospadias, ectopic or blind urethral opening
Pallister–Killian (Doray *et al*., 2002; Baglaj *et al*., 2008)	Tetrasomy 12p	30–40%	<5%	Cryptorchidism*
Cornelia de Lange (Kline *et al*., 2007)	*NIPBL* (most frequent), *SMC1A*, *SMC3*	<10%	55–75%	Cryptorchidism*, micropenis, hypospadias
Denys–Drash (Devriendt *et al*., 1995)	*WT1*	A few cases published	≈100%	Pseudohermaphroditism (ambiguous genitalia or female phenotype in males)

* Cryptorchidism in the context of CDH may be induced by decreased intra-abdominal pressure and should probably not be considered an independent malformation.

literature, and this syndrome was named Emanuel syndrome in 2004. Older case reports described this chromosome abnormality as 'partial trisomy 22' or 'partial trisomy 11'. The diaphragmatic defect associated with this abnormality has been attributed primarily to duplication of material from chromosome 22 according to the previously described cases of CDH associated with trisomy 22. This hypothesis has not been supported by the observation of absent CDH in babies with partial trisomy 22q resulting from a supernumerary der(22) with partial trisomy 8q secondary to 3 : 1 nondisjunction in families with translocation t(8;22). The breakpoints on the chromosome 22q are identical to those in the supernumerary der(22)t(11;22) (Mark *et al*., 2005). Recent observations of CDH in partial trisomy 11q due to an unbalanced translocation t(11;12) have suggested that duplication of one or more genes on a 19-megabase region of 11q23.3-qter confers predisposition to the development of CDH (Klaassens *et al*., 2006).

Fryns syndrome (FS) has been reported to be the most common multiple congenital anomaly syndrome associated with CDH (estimated frequency: 7 per 100 000 live births). The diagnosis is strongly suggested by the association of CDH with brachytelephalangy and/or nail hypoplasia after exclusion of chromosome aberrations (Slavotinek, 2004). Pulmonary hypoplasia, craniofacial

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dysmorphism and orofacial clefting are frequent and diagnostically useful. Other distinctive malformations include ventricular dilation or hydrocephalus, agenesis of the corpus callosum, neuronal or cerebellar heterotopias, anbormalities of the aorta, renal cysts, dilation of the ureters, bicornuate uterus, renal dysplasia, proximal thumbs and broad clavicles (Slavotinek, 2004). Genital anomalies are present in fewer than 20% of cases and consist of cryptorchidism, shawl scrotum, micropenis, ambiguous genitalia, hypospadias and ectopic or blind urethral opening (Slavotinek, 2004). Survival beyond the neonatal period is uncommon. The proposed autosomal recessive inheritance is based on the occurrence of parental consanguinity and affected sibling pairs. No genes or loci associated with FS have been identified or mapped.

Pallister–Killian syndrome (PKS) is a mosaic syndrome genetically defined as a tetrasomy of the short arm of chromosome 12 (Doray *et al*., 2002). The more consistent anomalies include CDH, rhizomelic micromelia and limb deformities, craniofacial dysmorphism (prominent forehead, flat occiput, hypertelorism, short nose with anteverted nases, low-set ears), cerebral anomalies, cardiovascular anomalies, anorectal anomalies and short neck (Doray *et al*., 2002; Baglaj *et al*., 2008). The main genital anomaly is cryptorchidism (Baglaj *et al*., 2008). Tetrasomy 12p may be diagnosed by chorionic villus cytogenetic analysis or by amniocentesis. Foetal blood analysis should be avoided because of the low level of the iso12p in this tissue (Doray *et al*., 2002). In perinatally diagnosed cases, a much higher incidence of internal organ anomalies is noted and is usually associated with a severely impaired phenotype including mental retardation (Doray *et al*., 2002; Baglaj *et al*., 2008).

Cornelia de Lange syndrome has been estimated to occur in about 1 in 10 000 individuals (Kline *et al*., 2007). Proportionate small stature persists throughout life starting prenatally. Facial features are the clinical hallmark of the syndrome and include hirsute forehead, synophrys and long eyelashes. Although almost any organ system can be affected, the neurodevelopmental, craniofacial, gastrointestinal and musculoskeletal systems are most commonly involved. Genital anomalies mainly comprise hypoplasia, cryptorchidism, micropenis and hypospadias (Kline *et al*., 2007). Ultrasound findings in the second trimester have demonstrated growth restriction, major malformations such as CDH, characteristic facial profile (flattened midface, short nose, anteverted nases, long philtrum, micrognathia) and hand and foot findings (shortened first metacarpal, brachydactyly, fifth finger clinodactyly, oligodactyly, absent forearm) (Kline *et al*., 2007). To date, three genes, *NIPBL*, *SMC1A* and *SMC3*, are known to be contributory, with approximately 50% of individuals displaying a detectable mutation, most often in *NIPBL* (Kline *et al*., 2007).

Denys–Drash syndrome is characterised by the association of diffuse mesangial sclerosis (DMS), pseudohermaphroditism (ambiguous genitalia or female phenotype with dysgenetic testis or streaked gonads) in all 46,XY patients and nephroblastoma. In contrast, all 46,XX children have a normal phenotype with normal ovaries. A few cases of CDH in the context of this syndrome have been described (Devriendt *et al*., 1995). Constitutive mutations in the *WT1* gene, mostly located in exons 8 and 9, have been described in the majority of patients with Denys–Drash syndrome. Progression to renal failure is constant within 1 to 4 years. Nephrotic syndrome does not recur after kidney transplantation.

For prenatally detected CDH with genital anomaly, foetal karyotyping can easily identify Emanuel syndrome and PKS. For normal karyotype, Fryns syndrome is the most common one.

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Annexes

Confection de sondes FISH à partir de clones BAC

1. Mise en culture sur milieu LB (Luria Bertani) solide

Ajouter 12,5 µg/µl de Chloramphénicol

Laisser une nuit à 37°C

Mettre à 4°C le lendemain matin

2. Mise en culture sur milieu LB liquide

Ajouter 12,5 µg/µl de Chloramphénicol

Laisser une nuit à 37°C avec agitation

3. Extraction de l'ADN : MiniPrep Kit NucleoSpin® Plasmid

4. Mesure de la densité optique

5. Marquage fluorescent de l'ADN par Nick Translation

Mix :

ADN (1µg)	x µl
NT enzyme[1]	10 µl
dNTP[1]	10 µl
dTTP[1] (0.1mM)	5 µl
10X NT Tampon[1]	5 µl
Spectrum dUTP[2] (0.2mM)	2.5 µl
H2O nucléase free	QSP 50 µl

[1]*Kit de Nick Translation (Abbott Molecular Inc)*

[2]*Vysis*

Thermocycleur : 2 heures à 15°C, 10 minutes à 70°C ; pause à 4°C

5. Préparation de la sonde

Mix :	réaction NT	8µl
	Cot1DNA[1]	1.6µl
	Acétate de Sodium	1.9µl
	H2O	1.7µl
	Ethanol 100% glacé	30µl

[1]*Abbott Molecular Inc*

Laisser incuber 15 min à 4°C à l'abri de la lumière

Centrifuger 12000 rpm 30 min, 4°C

Enlever le surnageant

Laisser sécher 5 à 10 min à l'abri de la lumière

Reprendre le culot avec 7µl de tampon LSI + 0.8µl de sonde contrôle

6. Préparation des lames

15 min dans pepsine, 37°C

5 min dans PBS, T^{re} ambiante

Déshydratation 2 min dans OH 70°C, 90°C et 100°C

Déposer 7-8 µl de sonde + lamelle 24*32

Hybrite : 2 min à 73°C puis 37°C toute la nuit

7. Lavages des lames

0,4 SSC à 73°C pendant 2 minutes

2 SSC à température ambiante pendant quelques secondes

Sécher

Déposer 15µl de DAPI + 1 goutte de Vectashield + 1 lamelle 24*32 + vernis

Mettre à 4°C pendant au moins 30 minutes

8. Lecture

Protocole MLPA
(MRC Holland)

1. Dénaturation et hybridation

120 ng d'ADN (6µl à 20ng/µl) par puit
Thermocycleur : 5 minutes à 98°C puis 25°
Ajouter le mix d'hybridation :
1,3 µl de sondes
1,7 µl de tampon
Thermocycleur : 1 minute à 95°C puis 16h à 60°C

2. Ligation

Mix : 3 µl de tampon A
3 µl de tampon B
25 µl d'H2O
1µl ligase
Thermocycleur : 15 minutes à 54°C puis 5 minutes à 98°C

3. PCR

Mix : 2µl de tampon de la réaction de PCR
1 µl amorces fluorescentes
1µl de tampon de dilution de l'enzyme
15,75 µl d'H2O
0,25 µl de polymérase
Thermocycleur :
35 cycles : 30 secondes à 95°C, 30 secondes à 60°C, 60 secondes à 72°C
puis 20 minutes à 72°C

4. Séparation des produits d'amplification par électrophorèse

(GeXP Beckman Coulter)

5. Interprétation des résultats grâce à un tableur Excel